

## **CHIMERIC IMMUNOMODULATORY COMPOUNDS AND METHODS OF USING THE SAME-IV**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

**[0001]** This application is a continuation-in-part application of patent application no. 10/328,578 which is a continuation-in-part of patent applications no. 10/176,883 and no. 10/177,826, both filed June 21, 2002, both of which claim benefit of provisional patent application no. 60/299,883, filed June 21, 2001 and provisional patent application no. 60/375,253, filed April 23, 2002. The entire contents of each of the aforementioned applications is incorporated herein by reference for all purposes.

### **FIELD OF THE INVENTION**

**[0002]** The present invention relates to chimeric immunomodulatory compounds ("CICs") containing nucleic acid and non-nucleic acid moieties, and to the use of such compounds to modulate an immune response. The invention finds use in the fields of biomedicine and immunology.

### **BACKGROUND**

**[0003]** Reference to a publication in this section should not be construed as an indication that the publication is prior art to the present invention.

**[0004]** The type of immune response generated by infection or other antigenic challenge can generally be distinguished by the subset of T helper (Th) cells involved in the response. The Th1 subset is responsible for classical cell-mediated functions such as delayed-type hypersensitivity and activation of cytotoxic T lymphocytes (CTLs), whereas the Th2 subset functions more effectively as a helper for B-cell activation. The type of immune response to an antigen is generally influenced by the cytokines produced by the cells responding to the antigen. Differences in the cytokines secreted by Th1 and Th2 cells are believed to reflect different biological

functions of these two subsets. See, for example, Romagnani (2000) *Ann. Allergy Asthma Immunol.* 85:9-18.

[0005] The Th1 subset may be particularly suited to respond to viral infections, intracellular pathogens, and tumor cells because it secretes IL-2 and IFN- $\gamma$ , which activate CTLs. The Th2 subset may be more suited to respond to free-living bacteria and helminthic parasites and may mediate allergic reactions, since IL-4 and IL-5 are known to induce IgE production and eosinophil activation, respectively. In general, Th1 and Th2 cells secrete distinct patterns of cytokines and so one type of response can moderate the activity of the other type of response. A shift in the Th1/Th2 balance can result in an allergic response, for example, or, alternatively, in an increased CTL response.

[0006] It has been recognized for some time that a Th1-type immune response can be induced in mammals by administration of certain immunomodulatory polynucleotides. The immunomodulatory polynucleotides include sequences referred to as immunostimulatory sequences ("ISS"), often including a CG. See, e.g., PCT Publications WO 98/55495, WO 97/28259, U.S. Pat. Nos. 6,194,388 and 6,207,646; and Krieg et al. (1995) *Nature* 374:546-49. For many infectious diseases, such as tuberculosis and malaria, Th2-type responses are of little protective value against infection. Protein-based vaccines typically induce Th2-type immune responses, characterized by high titers of neutralizing antibodies but without significant cell-mediated immunity. Moreover, some types of antibody responses are inappropriate in certain indications, most notably in allergy where an IgE antibody response can result in anaphylactic shock.

[0007] In view of the need for improved methods of immunotherapy, a need exists for identification of compounds for modulation of an immune response.

## BRIEF SUMMARY OF THE INVENTION

[0008] In an aspect, the invention is directed to a chimeric compound having immunomodulatory activity. The chimeric immunomodulatory compound ("CIC") generally comprises one or more nucleic acid moieties and one or more non-nucleic acid moieties. The nucleic acid moieties in a CIC with more than one nucleic acid moiety may be the same or different. The non-nucleic acid moieties in a CIC with more than one non-nucleic acid moiety may be the same or different. Thus, in one embodiment the CIC comprises two or more nucleic acid moieties and one or more non-nucleic acid spacer moieties, where at least one non-nucleic acid spacer moiety is covalently joined to two nucleic acid moieties. In an embodiment, at least one nucleic acid moiety comprises the sequence 5'-CG-3'. In an embodiment, at least one nucleic acid moiety comprises the sequence 5'-TCG-3'.

[0009] In one aspect, the invention provides a chimeric immunomodulatory compound that has a core structure with the formula " $N_1-S_1-N_2$ ", where  $N_1$  and  $N_2$  are nucleic acid moieties and  $S_1$  is a non-nucleic acid spacer moiety, and where the CIC exhibits immunomodulatory activity. In one embodiment, the core structure is " $N_1-S_1-N_2-S_2-N_3$ ", where  $N_3$  is a nucleic acid moiety and  $S_2$  is a non-nucleic acid spacer moiety. In one embodiment, the CIC has the core structure " $N_1-S_1-N_2-S_2-[N_v-S_v]_A$ ", where  $A$  is an integer between 1 and 100, and  $[N_v-S_v]_A$  indicates  $A$  additional iterations of nucleic acid moieties conjugated to non-nucleic acid spacer moieties, where  $S$  and  $N$  are independently selected in each iteration of " $[N_v-S_v]$ ". In an embodiment,  $A$  is at least 2, and at least 4 nucleic acid moieties in the CIC have different sequences.

[0010] In an aspect, the CIC comprises a core structure with the formula  $N_1-S_1-N_2$  or  $N_1-S_1-N_2-S_2-N_3$  (wherein  $N_1$ ,  $N_2$ , and  $N_3$  are nucleic acid moieties,  $S_1$  and  $S_2$  are non-nucleic acid spacer moieties, and  $S_1$  and  $S_2$  are covalently bound to exactly two nucleic acid moieties). Examples of such CICs are CICs with core structures of the formula (5'- $N_1$ -3')- $S_1$ - $N_2$ . In one embodiment,  $N_1$  has the sequence 5'-TCGAX-3',

wherein X is 0 to 20 nucleotide bases (SEQ ID NO:1). In one embodiment, X is 0 to 3 nucleotide bases. In one embodiment, X is CGT. In another embodiment N<sub>1</sub> has the sequence 5'-TCGTCGA-3'. In an embodiment, the CIC has the structure N<sub>1</sub>-S<sub>1</sub>-N<sub>2</sub>-S<sub>2</sub>-[N<sub>v</sub>-S<sub>v</sub>]<sub>A</sub> (wherein A is an integer between 1 and 100, and [N<sub>v</sub>-S<sub>v</sub>]<sub>A</sub> indicates "A" additional iterations of nucleic acid moieties conjugated to non-nucleotide spacer moieties, where S and N are independently selected in each iteration of [N<sub>v</sub>-S<sub>v</sub>]). In an embodiment, A is 1 to 3.

**[0011]** In another aspect, the invention provides a CIC that has a core structure with the formula [N<sub>v</sub>]<sub>A</sub>---S<sub>p</sub>, where S<sub>p</sub> is a multivalent spacer covalently bonded to the quantity "A" independently selected nucleic acid moieties, N<sub>v</sub>, where A is at least 3, and where the CIC exhibits immunomodulatory activity. In one embodiment, the CIC has the core sequence [S<sub>v</sub>-N<sub>v</sub>]<sub>A</sub>---S<sub>p</sub> where S<sub>p</sub> is a multivalent spacer covalently bonded to the quantity "A" independently selected elements [S<sub>v</sub>-N<sub>v</sub>], and independently selected element [S<sub>v</sub>-N<sub>v</sub>] includes a spacer moiety covalently bound to a nucleic acid moiety, and wherein A is at least 3. In one embodiment, A is from 3 to 50. In a different embodiment, A is greater than 50. In an embodiment, at least 2, at least 3 or at least 4 nucleic acid moieties in the CIC have different sequences.

**[0012]** In an aspect, the CIC comprises a core structure with the formula [N<sub>v</sub>]<sub>A</sub>-S<sub>p</sub> or [S<sub>v</sub>-N<sub>v</sub>]<sub>A</sub>-S<sub>p</sub> (where S<sub>p</sub> is a multivalent spacer covalently bonded to the quantity "A" independently selected nucleic acid moieties, N<sub>v</sub>, or independently selected elements [S<sub>v</sub>-N<sub>v</sub>], each independently selected element [S<sub>v</sub>-N<sub>v</sub>] comprising a spacer moiety covalently bound to a nucleic acid moiety, wherein A is at least 3. In embodiments, A is from 3 to about 50 or from about 50 to about 500. In an embodiment, S<sub>p</sub> comprises a dendrimer. In an embodiment, a nucleic acid moiety of the CIC has a sequence selected from TCGXXXX, TCGAXXX, XTCGXXX, XTCGAXX, TCGTCGA, TCGACGT, TCGAACG, TCGAGAT, TCGACTC, TCGAGCG, TCGATTT, TCGCTTT, TCGGTTT, TCGTTTT, TCGTCGT, ATCGATT, TTCGTTT, TTCGATT, ACGTTCG, AACGTTC, TGACGTT,



TGTCGTT, TCGXXX, TCGAXX, TCGTCG, AACGTT, ATCGAT, GTCGTT, GACGTT, TCGXX, TCGAX, TCGAT, TCGTT, TCGTC, TCGA, TCGT, TCGX, or TCG (where "X" is any nucleotide).

**[0013]** In another aspect, the invention provides a CIC that has a core structure with the formula " $N_1-S_1$ ", where  $N_1$  is a nucleic acid moiety and  $S_1$  is a non-nucleic acid spacer moiety, and the CIC exhibits immunomodulatory activity.

**[0014]** The CIC may comprise non-nucleotide spacer moieties comprising, for example, triethylene glycol, hexaethylene glycol, a polymer comprising phosphodiester and/or phosphorothioate linked oligoethylene glycol moieties,  $C_2$ - $C_{10}$  alkyl (e.g., propyl, butyl, hexyl), glycerol or a modified glycerol (e.g., glycerol derivatized at the 1, 2 or 3 hydroxy-position; e.g., by addition of an alkylether), pentaerythritol or modified pentaerythritol (pentaerythritol modified at any hydroxy position(s), e.g., "trebler"), 2-(hydroxymethyl)ethyl, 1,3-diamino-2-propanol or modified 1,3-diamino-2-propanol (e.g., "symmetrical doubler" [Glen Research]), an abasic nucleotide, a polysaccharide (e.g., a cross-linked polysaccharide), a dendrimer, and/or other spacer moiety components disclosed herein, in various combinations.

**[0015]** In a related aspect, the invention provides a CIC that is not a branched CIC and which includes two nucleic acid moieties that are at least partially complementary to each other and able to form a duplex structure. In exemplary embodiments, at least one of the nucleic acid moieties includes the sequence 5'-TCG-3', 5'-TCGA-3', 5'-TCGACGT-3' or 5'-TCGTCGA-3, optionally in the 5-prime position.

**[0016]** In a related aspect, the invention provides a branched CIC that has a fork structure, an H structure, a comb structure, or a central spacer structure. In specific embodiments, a non-nucleic acid spacer moiety of the CIC includes a glycerol component and/or an oligoethylene glycol component (e.g., HEG). In an embodiment, the non-nucleic acid spacer moiety is a compound spacer. In exemplary embodiments, at least one of the nucleic acid moieties includes the sequence 5'-TCG-

3', 5'-TCGA-3', 5'-TCGACGT-3' or 5'-TCGTCGA-3, optionally in the 5-prime position.

**[0017]** In a related aspect, the invention provides a multimeric CIC including a first CIC and a second CIC, where the first CIC is not a branched CIC, and the second CIC is or is not a branched CIC, where a nucleic acid moiety of the first CIC is at least partially complementary to a nucleic acid moiety of the second CIC, and where the two nucleic acid moieties form a duplex structure. In an embodiment, both the first and second CICs is a branched CIC. In an embodiment, one or both the first and second CIC has a fork structure, an H structure, a comb structure, or a central spacer structure. In an embodiment, the multimeric CIC has a central axis structure or a cage structure. In exemplary embodiments, at least one nucleic acid moiety in one or more of the CICs of the multimeric CIC includes the sequence 5'-TCG-3', 5'-TCGA-3', 5'-TCGACGT-3' or 5'-TCGTCGA-3, optionally in the 5-prime position. In an embodiment, all of the nucleic acid moieties, or all of 5-prime moieties, in one, two or more of the CICs of the multimeric CIC have the same sequence.

**[0018]** In various embodiments, a CIC described above has one or more of the following characteristics: (i) the CIC includes at least one nucleic acid moiety less than 8 nucleotides (or base pairs) in length, or, alternatively, less than 7 nucleotides in length (ii) all of the nucleic acid moieties of the CIC are less than 8 nucleotides in length, or, alternatively, less than 7 nucleotides in length, (iii) the CIC includes at least one nucleic acid moiety that includes the sequence 5'-CG-3' (e.g., 5'-TCG-3'), (iv) the CIC includes at least two nucleic acid moieties having different sequences, (v) all of the nucleic acid moieties of the CIC have the same sequence, (vi) the CIC includes at least one non-nucleic acid spacer moiety that is or comprises triethylene glycol, hexaethylene glycol, propyl, butyl, hexyl, glycerol or a modified glycerol (e.g., glycerol derivatized at the 1, 2 or 3 hydroxy-position; e.g., by addition of an alkylether), pentaerythritol or modified pentaerythritol (pentaerythritol modified at any hydroxy position(s), e.g., "trebler"), 2-(hydroxymethyl)ethyl, 1,3-diamino-2-

propanol or modified 1,3-diamino-2-propanol (e.g., "symmetrical doubler" [Glen Research]), an abasic nucleotide, a polysaccharide (e.g., a cross-linked polysaccharide), or a dendrimer.

**[0019]** In various embodiments, a CIC described herein has one or more of the following characteristics: (vii) the CIC includes at least one nucleic acid moiety of the CIC that does not have "isolated immunomodulatory activity," (viii) the CIC does not include any nucleic acid moiety with "isolated immunomodulatory activity," (ix) the CIC includes at least one nucleic acid moiety of the CIC that has "inferior isolated immunological activity." "Isolated immunomodulatory activity" and "inferior isolated immunological activity" are described herein. In various embodiments a CIC described herein includes at least one nucleic acid moiety that is double-stranded or partially double-stranded. CICs can be designed with self-complementary nucleic acid moieties such that duplexes can be formed. See, e.g., C-84, C-85, and C-87.

**[0020]** Thus, in various aspects, the invention provides a CIC comprising two or more nucleic acid moieties and one or more non-nucleic acid spacer moieties, wherein at least one spacer moiety is covalently joined to two nucleic acid moieties and at least one nucleic acid moiety comprises the sequence 5'-CG-3', and wherein said CIC has immunomodulatory activity. The CIC may comprise at least three nucleic acid moieties, wherein each nucleic acid moiety is covalently joined to at least one non-nucleic acid spacer moiety. The CIC may have at least one immunomodulatory activity such as (a) the ability to stimulate IFN- $\gamma$  production by human peripheral blood mononuclear cells; (b) the ability to stimulate IFN- $\alpha$  production by human peripheral blood mononuclear cells; and/or (c) the ability to stimulate proliferation of human B cells.

**[0021]** One or more nucleic acid moieties of the CIC can comprise a sequence such as 5'-TCGA-3', 5'-TCGACGT-3', 5'-TCGTCGA-3' and 5'-ACGTTCG-3'. In an embodiment, one or more nucleic acid moieties of the CIC can have the sequence 5'-X<sub>1</sub>X<sub>2</sub>CGX<sub>3</sub>X<sub>4</sub>-3' (where X<sub>1</sub> is zero to ten nucleotides; X<sub>2</sub> is absent or is A, T, or U;

X<sub>3</sub> is absent or is A; and X<sub>4</sub> is zero to ten nucleotides, and wherein the nucleic acid moiety is conjugated to a spacer moiety, for example at the 3' end). In an embodiment, the sum of nucleotides in X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, and X<sub>4</sub> can be less than 8, less than 7, less than 6, less than 5 or less than 4. In some embodiments, one or more nucleic acid moieties of the CIC can have a nucleic acid sequence such as TCGXXXX, TCGAXXX, XTCGXXX, XTCGAXX, TCGTCGA, TCGACGT, TCGAACG, TCGAGAT, TCGACTC, TCGAGCG, TCGATTT, TCGCTTT, TCGGTTT, TCGTTTT, TCGTCGT, ATCGATT, TTCGTTT, TTCGATT, ACGTTCG, AACGTTC, TGACGTT, TGTCGTT, TCGXXX, TCGAXX, TCGTCG, AACGTT, ATCGAT, GTCGTT, GACGTT, TCGXX, TCGAX, TCGAT, TCGTT, TCGTC, TCGA, TCGT, TCGX, or TCG (where "X" is any nucleotide).

**[0022]** In one embodiment, one or more nucleic acid moieties comprises 3 to 7 bases. In one embodiment, the nucleic acid moiety comprises 3 to 7 bases and has the sequence 5'-[(X)<sub>0-2</sub>]TCG[(X)<sub>2-4</sub>]-3', or 5'-TCG[(X)<sub>2-4</sub>]-3', or 5'-TCG(A/T)[(X)<sub>1-3</sub>]-3', or 5'-TCG(A/T)CG(A/T)-3', or 5'-TCGACGT-3' or 5'-TCGTCGA-3', wherein each X is an independently selected nucleotide. In some embodiments, the CIC contains at least 3, at least 10, at least 30 or at least 100 nucleic acid moieties having a sequence described above.

**[0023]** In one aspect, the invention provides a chimeric immunomodulatory compound (CIC) that stimulates production of IFN- $\alpha$  from human peripheral blood mononuclear cells and has at least three nucleic acid moieties and at least one nonnucleic acid spacer moiety, where at least one nucleic acid moiety comprises a motif 5'-TCGXCGX 5'-TCGXTCG 5'-TCGXXCG, or 5'-TCGCGXX, where X is any nucleotide (for example, 5'<sup>F</sup>-TCGXCGX 5'<sup>F</sup>-TCGXTCG, 5'<sup>F</sup>-TCGXXCG, or 5'<sup>F</sup>-TCGCGXX). The CIC may have any of the structures described herein for CICs and, for example, may comprise at least one multivalent nonnucleic acid spacer moiety and/or may comprise at least one nonnucleic acid spacer moiety comprising

HEG, TEG, propyl, butyl, hexyl, pentaerythritol, 2-(hydroxymethyl)ethyl, glycerol, a polysaccharide, 1,3-diamino-2-propanol, or a dendrimer.

**[0024]** The CIC can include at least one nucleic acid moiety that is less than 8 nucleotides in length. Optionally all the nucleic acid moieties in the CIC are less than 8 nucleotides in length. In some embodiments, all the nucleic acid moieties in the CIC that comprise the sequence 5'-CG-3' are less than 8 nucleotides in length. The CIC can include at least 2 nucleic acid moieties having different sequences. The CIC can contain at least one nucleic acid moiety does not comprise the sequence 5'-CG-3'. The CIC may include at least one nucleic acid moiety that does not have isolated immunological activity or has inferior isolated immunological activity. Optionally no nucleic acid moiety of the CIC has isolated immunomodulatory activity. The linkages between the nucleotides of the nucleic acid moieties may include phosphodiester, phosphorothioate ester, phosphorodithioate ester, other covalent linkages, and mixtures thereof. Similarly, the linkages between nucleic acid moieties and spacer moieties or between components of spacer moieties may include phosphodiester, phosphorothioate ester, phosphorodithioate ester, other linkages, and mixtures thereof.

**[0025]** In an embodiment, the CIC includes a reactive linking group (e.g., a reactive thio group). The CIC may be linked or noncovalently associated with a polypeptide, e.g., a polypeptide antigen.

**[0026]** The invention also provides compositions comprising a CIC along with a pharmaceutically acceptable excipient and/or an antigen and/or a cationic microcarrier (such as a polymer of lactic acid and glycolic acid). The composition can be essentially endotoxin-free.

**[0027]** In an aspect, the invention provides a composition containing a CIC described herein and a pharmaceutically acceptable excipient, an antigen (e.g., an antigen to which an immune response is desired), or both. In an embodiment, the composition is formulated under GMP standards. In an embodiment, the composition

is prepared by a process that includes assaying the composition for the presence of endotoxin. In an embodiment, the composition is essentially endotoxin-free. In an embodiment, the composition does not contain liposomes.

[0028] In an aspect, the invention provides the use of a CIC as described herein for the manufacture of a medicament.

[0029] In an aspect, the invention provides a method of modulating an immune response of a cell by contacting the cell with a CIC-containing composition. In an embodiment, the CIC-containing composition comprises a multimeric CIC.

[0030] In an aspect, the invention provides a method of modulating an immune response in an individual by administering a chimeric immunomodulatory compound or CIC-containing composition as described herein, in an amount sufficient to modulate an immune response in the individual. In one embodiment, the individual suffers from a disorder associated with a Th2-type immune response, for example, an allergy or allergy-induced asthma. In one embodiment, the individual has an infectious disease.

[0031] In an aspect, the invention provides a method of increasing interferon-gamma (IFN- $\gamma$ ) in an individual by administering a CIC or composition as described herein, in an amount sufficient to increase IFN- $\gamma$  in the individual. In an embodiment, the individual has an inflammatory disorder. In an embodiment, the individual has idiopathic pulmonary fibrosis.

[0032] In an aspect, the invention provides a method of increasing interferon-alpha (IFN- $\alpha$ ) in an individual, by administering a CIC or composition as described herein, in an amount sufficient to increase IFN- $\alpha$  in the individual. In an embodiment, the individual has a viral infection.

[0033] In one aspect, the invention provides a CIC that stimulates production of IFN- $\alpha$  from human peripheral blood mononuclear cells but does not stimulate human B cell proliferation, or stimulates little B cell proliferation. For example, but not limitation, this CIC may comprise a nucleic acid moiety comprising the sequence 5'-

TCGAX<sub>N</sub>, (for example, 5'<sup>F</sup>-TCGAX<sub>N</sub>) where X is any nucleotide and n is 1, 2, or 3; a nucleic acid moiety comprising the sequence 5'-TCGAX<sub>N</sub>, where X is any amino acid and n is an integer from 4 to 9; or a nucleic acid moiety comprising the sequence 5'-TCGACGX<sub>N</sub>, (for example, 5'<sup>F</sup>-TCGACGX<sub>N</sub>) where X is any nucleotide and n is 1 or, alternatively, n is 2, 3, or an integer from 4 to 7. In an embodiment, X is T (5'-TCGACGT). For example, but not limitation, the CIC may have the structure (5'-TCGACGT-HEG)<sub>2</sub>-glycerol-HEG-5'-TCGACGT or (5'-TCGACGT-HEG)<sub>3</sub>-treble-HEG-5'-TCGACGT.

[0034] In a related aspect, the invention provides a composition comprising a CIC that stimulates production of IFN- $\alpha$  from human peripheral blood mononuclear cells but does not stimulate human B cell proliferation, or stimulates little human B cell proliferation, and a pharmaceutically acceptable excipient. In related embodiments the composition also includes an antigen and/or a cationic microsphere (e.g. as described herein).

[0035] In an aspect, the invention provides a method of ameliorating a symptom of an infectious disease in an individual, by administering an effective amount of a CIC or composition, as described herein, to the individual, where the effective amount is an amount sufficient to ameliorate a symptom of the infectious disease.

[0036] In an aspect, the invention provides a method of ameliorating an IgE-related disorder in an individual, by administering an effective amount of a CIC or composition described herein to an individual having an IgE-related disorder, where an effective amount is an amount sufficient to ameliorate a symptom of the IgE-related disorder. In an embodiment, the IgE-related disorder is allergy or an allergy-related disorder.

[0037] The invention further provides a method of modulating an immune response in an individual by administering to an individual a CIC in an amount sufficient to modulate an immune response in said individual. In embodiments, the individual has cancer and/or suffers from a disorder associated with a Th2-type

immune response (e.g., an allergy or allergy-induced asthma) and/or has an infectious disease.

### BRIEF DESCRIPTION OF THE FIGURES

[0038] Figure 1 shows the structure of certain reagents useful for synthesis of non-nucleic acid spacer moieties of CICs. Shown are dimethoxytrityl-protected phosphoramidite spacer-moiety precursors for HEG, propyl, TEG, HME, butyl, and abasic spacer moieties.

[0039] Figure 2 shows the structure of certain reagents useful for synthesis of symmetric or asymmetric non-nucleic acid spacer moieties of CICs. Shown are dimethoxytrityl-protected phosphoramidite spacer moiety precursors for glycerol [2] "symmetrical branched"), levulinyl-glycerol [3] ("asymmetrical branched"), "trebler" [9] and "symmetrical doubler" [10] spacer moieties.

[0040] Figures 3A and 3B diagram the synthesis of a branched CIC.

[0041] Figure 4 shows the synthetic scheme for C-105.

[0042] Figure 5 shows induction of immune-associated genes in the mouse lung after intranasal treatment with CICs.

[0043] Figures 6A-C show the effect of CICs on levels of IL-12 p40 (Fig. 6A), IL-6 (Fig. 6B), and TNF-alpha (Fig. 6C).

[0044] Figures 7A-B show the structures of C-8 (Fig. 7A) and C-101 (Fig. 7B).

[0045] Figures 8A-8H show examples of CICs having defined secondary or tertiary structure. Fig. 8A shows a linear CIC having the structure of a hairpin duplex; Fig. 8B shows a branched CIC having a "fork" structure; Fig. 8C shows a branched CIC with an "H" structure; Fig. 8D shows a branched CIC with a "comb" structure; Fig. 8E shows a branched CIC with a "central-spacer" structure; Fig. 8F shows a branched CIC with a "central-spacer" structure; Fig. 8G illustrates synthesis of a branched CIC with a "central-spacer" structure by a conjugation strategy; Fig. 8H shows a CIC dendrimer. (H=HEG; N=1-5; A = 5' adenosine; G = 5' guanosine;



“|” indicates base-pairing). The sequence identifier for the sequence shown in Fig. 8A is ATCGATCGTTCGAGCGAC (SEQUENCE ID NO:140).

**[0046]** Figures 9A-9G show examples of CIC multimers. Fig. 9A shows a CIC multimer having the structure of a linear CIC duplex and comprising two identical CICs; Fig. 9B shows a CIC multimer having the structure of a linear CIC duplex and comprising two different CICs; Fig. 9C shows a linear dimer having 5' ends that are not base-paired; Fig. 9D shows a CIC multimer having the structure of a concatamer of five linear CICs; Fig. 9E shows a CIC multimer with a “central axis” structure; Fig. 9F shows a CIC multimer with a “cage” structure; Fig. 9G shows a CIC multimer with a “starfish” structure. (H=HEG; A = 5' adenosine; T = 5' thymidine; G = 5' guanosine). The sequence identifiers for sequences shown in Figures 9A-9G are: ATCGATCGTTCGAGCGAC (SEQ ID NO:140); GTCGCTCGAACGATCGAT (SEQ ID NO:141); AGGGTTTTTTTTTTTTT (SEQ ID NO:142); TCGATCGATCGATCGTTCGAGCGAC (SEQ ID NO:143); GTCGCTCGAACGATCGATTAAACAAAC (SEQ ID NO:144); GTCGCTCGAACGATCGATAATAAAT (SEQ ID NO:145); TCGATCGTTATCGATCGTTCGAGCGAC (SEQ ID NO:146); TCGATTTCGAGCG (SEQ ID NO:147); TCGTTCGAGCGAATTCGCTCGAACGATCTT (SEQ ID NO:148); TCGTTTTTTTTTCGC (SEQ ID NO:149); AAAAAAACGCCG (SEQ ID NO:150); TCGCGAAAAAAACGA (SEQ ID NO:151); ATCATCCGAACGTTGA (SEQ ID NO:152).

**[0047]** Figure 10 shows effects of CIC structure, spacer composition, and NAM sequence on IFN- $\alpha$  and IFN- $\gamma$  production. PBMCs were isolated from 8 donors and stimulated with 20  $\mu$ g/ml P-6 or CIC for 24 h. Cell-free supernatants were assayed for IFN- $\gamma$  and IFN- $\alpha$  content by ELISA. Data are shown as means  $\pm$  SEM. Statistical relevance: \*\*,  $p < 0.01$ , \*,  $p < 0.05$ , where P-6 and the linear CICs (C-74, C-75, C-76, C-77, C-73, C-41, C-21 and C-51) were compared to the linear chimeric control

ODN, M-2 and branched CICs ( C-143, C-94, C-101, C-142, C-103, C-104, C-28, C-145, C-156, C-157, C-158, and C-125) were compared to the branched chimeric control ODN, M-3.

[0048] Figure 11 shows the effect of NAM sequence (motifs) on the level of human B cell activity. Purified human B cells from 2 donors were stimulated with 5 µg/ml P-6 or CIC for 96 h. Proliferation was assessed by <sup>3</sup>H-thymidine incorporation. This assay is representative of two separate assays with two donors each.

## DETAILED DESCRIPTION OF THE INVENTION

### I. General Methods

[0049] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989) and *Molecular Cloning: A Laboratory Manual*, third edition (Sambrook and Russel, 2001), (jointly and individually referred to herein as "Sambrook"). *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Handbook of Experimental Immunology* (D.M. Weir & C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987, including supplements through 2001); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); *The Immunoassay Handbook* (D. Wild, ed., Stockton Press NY, 1994); *Bioconjugate Techniques* (Greg T. Hermanson, ed., Academic Press, 1996); *Methods of Immunological Analysis* (R. Masseyeff, W.H. Albert, and N.A. Staines, eds., Weinheim: VCH Verlags gesellschaft mbH, 1993), Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, and

Harlow and Lane (1999) *Using Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (jointly and individually referred to herein as "Harlow and Lane"), Beaucage et al. eds., *Current Protocols in Nucleic Acid Chemistry* John Wiley & Sons, Inc., New York, 2000); and Agrawal, ed., *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* Humana Press Inc., New Jersey, 1993).

## II. Definitions

**[0050]** As used herein, the singular form "a", "an", and "the" includes plural references unless otherwise indicated or clear from context. For example, as will be apparent from context, "a" chimeric immunomodulatory/immunostimulatory compound ("CIC") can include one or more CICs. Similarly, reference in the singular form of a component element of a CIC (i.e., nucleic acid moiety or non-nucleic acid spacer moiety) can include multiple elements. For example, a description of "a nucleic acid moiety" in a CIC can also describe two or more "nucleic acid moieties" in the CIC.

**[0051]** As used interchangeably herein, the terms "polynucleotide," "oligonucleotide" and "nucleic acid" include single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), modified oligonucleotides and oligonucleosides, or combinations thereof. The nucleic acid can be linearly or circularly configured, or the oligonucleotide can contain both linear and circular segments. Nucleic acids are polymers of nucleosides joined, e.g., through phosphodiester linkages or alternate linkages, such as phosphorothioate esters. A nucleoside consists of a purine (adenine (A) or guanine (G) or derivative thereof) or pyrimidine (thymine (T), cytosine (C) or uracil (U), or derivative thereof) base bonded to a sugar. The four nucleoside units (or bases) in DNA are called deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. A nucleotide is a phosphate ester of a nucleoside.

[0052] The term "3'" generally refers to a region or position in a polynucleotide or oligonucleotide 3' (downstream) from another region or position in the same polynucleotide or oligonucleotide.

[0053] The term "5'" generally refers to a region or position in a polynucleotide or oligonucleotide 5' (upstream) from another region or position in the same polynucleotide or oligonucleotide.

[0054] An element, *e.g.*, region, portion, non-nucleic acid spacer moiety, nucleic acid moiety, or sequence is "adjacent" to another element, *e.g.*, region, portion, non-nucleic acid spacer moiety, nucleic acid moiety, or sequence, when it directly abuts that region, portion, spacer or sequence.

[0055] The term "CIC-antigen conjugate" refers to a complex in which a CIC and an antigen are linked. Such conjugate linkages include covalent and/or non-covalent linkages.

[0056] The term "antigen" means a substance that is recognized and bound specifically by an antibody or by a T cell antigen receptor. Antigens can include peptides, proteins, glycoproteins, polysaccharides, complex carbohydrates, sugars, gangliosides, lipids and phospholipids; portions thereof and combinations thereof. The antigens can be those found in nature or can be synthetic. Antigens suitable for administration with a CIC includes any molecule capable of eliciting a B cell or T cell antigen-specific response. Preferably, antigens elicit an antibody response specific for the antigen. Haptens are included within the scope of "antigen." A hapten is a low molecular weight compound that is not immunogenic by itself but is rendered immunogenic when conjugated with an immunogenic molecule containing antigenic determinants. Small molecules may need to be haptenized in order to be rendered antigenic. Preferably, antigens of the present invention include peptides, lipids (*e.g.* sterols, fatty acids, and phospholipids), polysaccharides such as those used in Hemophilus influenza vaccines, gangliosides and glycoproteins.

[0057] "Adjuvant" refers to a substance which, when added to an immunogenic agent such as antigen, nonspecifically enhances or potentiates an immune response to the agent in the recipient host upon exposure to the mixture.

[0058] The term "peptide" are polypeptides that are of sufficient length and composition to effect a biological response, *e.g.*, antibody production or cytokine activity whether or not the peptide is a hapten. Typically, the peptides are at least six amino acid residues in length. The term "peptide" further includes modified amino acids (whether or not naturally or non-naturally occurring), such modifications including, but not limited to, phosphorylation, glycosylation, pegylation, lipidization and methylation.

[0059] "Antigenic peptides" can include purified native peptides, synthetic peptides, recombinant peptides, crude peptide extracts, or peptides in a partially purified or unpurified active state (such as peptides that are part of attenuated or inactivated viruses, cells, micro-organisms), or fragments of such peptides. An "antigenic peptide" or "antigen polypeptide" accordingly means all or a portion of a polypeptide which exhibits one or more antigenic properties. Thus, for example, an "Amb a 1 antigenic polypeptide" or "Amb a 1 polypeptide antigen" is an amino acid sequence from Amb a 1, whether the entire sequence, a portion of the sequence, and/or a modification of the sequence, which exhibits an antigenic property (*i.e.*, binds specifically to an antibody or a T cell receptor).

[0060] A "delivery molecule" or "delivery vehicle" is a chemical moiety which facilitates, permits, and/or enhances delivery of a CIC, CIC-antigen mixture, or CIC-antigen conjugate to a particular site and/or with respect to particular timing. A delivery vehicle may or may not additionally stimulate an immune response.

[0061] An "allergic response to antigen" means an immune response generally characterized by the generation of eosinophils (usually in the lung) and/or antigen-specific IgE and their resultant effects. As is well-known in the art, IgE binds to IgE receptors on mast cells and basophils. Upon later exposure to the antigen recognized

by the IgE, the antigen cross-links the IgE on the mast cells and basophils causing degranulation of these cells, including, but not limited, to histamine release. It is understood and intended that the terms “allergic response to antigen”, “allergy”, and “allergic condition” are equally appropriate for application of some of the methods of the invention. Further, it is understood and intended that the methods of the invention include those that are equally appropriate for prevention of an allergic response as well as treating a pre-existing allergic condition.

**[0062]** As used herein, the term “allergen” means an antigen or antigenic portion of a molecule, usually a protein, which elicits an allergic response upon exposure to a subject. Typically the subject is allergic to the allergen as indicated, for instance, by the wheal and flare test or any method known in the art. A molecule is said to be an allergen even if only a small subset of subjects exhibit an allergic (e.g., IgE) immune response upon exposure to the molecule. A number of isolated allergens are known in the art. These include, but are not limited to, those provided in Table 1 herein.

**[0063]** The term “desensitization” refers to the process of the administration of increasing doses of an allergen to which the subject has demonstrated sensitivity. Examples of allergen doses used for desensitization are known in the art, see, for example, Fornadley (1998) *Otolaryngol. Clin. North Am.* 31:111-127.

**[0064]** “Antigen-specific immunotherapy” refers to any form of immunotherapy which involves antigen and generates an antigen-specific modulation of the immune response. In the allergy context, antigen-specific immunotherapy includes, but is not limited to, desensitization therapy.

**[0065]** The term “microcarrier” refers to a particulate composition which is insoluble in water and which has a size of less than about 150, 120 or 100  $\mu\text{m}$ , more commonly less than about 50-60  $\mu\text{m}$ , and may be less than about 10  $\mu\text{m}$  or even less than about 5  $\mu\text{m}$ . Microcarriers include “nanocarriers”, which are microcarriers have a size of less than about 1  $\mu\text{m}$ , preferably less than about 500 nm. Microcarriers include solid phase particles such as particles formed from biocompatible naturally

occurring polymers, synthetic polymers or synthetic copolymers, although microcarriers formed from agarose or cross-linked agarose may be included or excluded from the definition of microcarriers herein as well as other biodegradable materials known in the art. Solid phase microcarriers are formed from polymers or other materials which are non-erodible and/or non-degradable under mammalian physiological conditions, such as polystyrene, polypropylene, silica, ceramic, polyacrylamide, gold, latex, hydroxyapatite, and ferromagnetic and paramagnetic materials. Biodegradable solid phase microcarriers may be formed from polymers which are degradable (*e.g.*, poly(lactic acid), poly(glycolic acid) and copolymers thereof, such as poly(D, L-lactide-co-glycolide) or erodible (*e.g.*, poly(ortho esters such as 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5.5] undecane (DETOSU) or poly(anhydrides), such as poly(anhydrides) of sebacic acid) under mammalian physiological conditions. Microcarriers are typically spherical in shape, but microcarriers which deviate from spherical shape are also acceptable (*e.g.*, ellipsoidal, rod-shaped, etc.). Due to their insoluble nature, solid phase microcarriers are filterable from water and water-based (aqueous) solutions (*e.g.*, using a 0.2 micron filter). Microcarriers may also be liquid phase (*e.g.*, oil or lipid based), such as liposomes, iscoms (immune-stimulating complexes, which are stable complexes of cholesterol, phospholipid and adjuvant-active saponin) without antigen, or droplets or micelles found in oil-in-water or water-in-oil emulsions. Biodegradable liquid phase microcarriers typically incorporate a biodegradable oil, a number of which are known in the art, including squalene and vegetable oils. The term "nonbiodegradable", as used herein, refers to a microcarrier which is not degraded or eroded under normal mammalian physiological conditions. Generally, a microcarrier is considered nonbiodegradable if it not degraded (*i.e.*, loses less than 5% of its mass or average polymer length) after a 72 hour incubation at 37° C in normal human serum.

[0066] A microcarrier is considered "biodegradable" if it is degradable or erodable under normal mammalian physiological conditions. Generally, a microcarrier is considered biodegradable if it is degraded (*i.e.*, loses at least 5% of its mass or average polymer length) after a 72 hour incubation at 37° C in normal human serum.

[0067] The term "CIC/microcarrier complex" or "CIC/MC complex" refers to a complex of a CIC and a microcarrier. The components of the complex may be covalently or non-covalently linked. Non-covalent linkages may be mediated by any non-covalent bonding force, including by hydrophobic interaction, ionic (electrostatic) bonding, hydrogen bonds and/or van der Waals attractions. In the case of hydrophobic linkages, the linkage is generally via a hydrophobic moiety (*e.g.*, cholesterol) covalently linked to the CIC.

[0068] An "individual" or "subject" is a vertebrate, such as avian, preferably a mammal, such as a human. Mammals include, but are not limited to, humans, non-human primates, farm animals, sport animals, experimental animals, rodents (*e.g.*, mice and rats) and pets.

[0069] An "effective amount" or a "sufficient amount" of a substance is that amount sufficient to effect a desired biological effect, such as beneficial results, including clinical results, and, as such, an "effective amount" depends upon the context in which it is being applied. In the context of administering a composition that modulates an immune response to a co-administered antigen, an effective amount of a CIC and antigen is an amount sufficient to achieve such a modulation as compared to the immune response obtained when the antigen is administered alone. An effective amount can be administered in one or more administrations.

[0070] The term "co-administration" as used herein refers to the administration of at least two different substances sufficiently close in time to modulate an immune response. Preferably, co-administration refers to simultaneous administration of at least two different substances.



[0071] "Stimulation" of an immune response, such as Th1 response, means an increase in the response, which can arise from eliciting and/or enhancement of a response. Similarly, "stimulation" of a cytokine or cell type (such as CTLs) means an increase in the amount or level of cytokine or cell type.

[0072] An "IgE associated disorder" is a physiological condition which is characterized, in part, by elevated IgE levels, which may or may not be persistent. IgE associated disorders include, but are not limited to, allergy and allergic reactions, allergy-related disorders (described below), asthma, rhinitis, atopic dermatitis, conjunctivitis, urticaria, shock, *Hymenoptera* sting allergies, food allergies, and drug allergies, and parasite infections. The term also includes related manifestations of these disorders. Generally, IgE in such disorders is antigen-specific.

[0073] An "allergy-related disorder" means a disorder resulting from the effects of an antigen-specific IgE immune response. Such effects can include, but are not limited to, hypotension and shock. Anaphylaxis is an example of an allergy-related disorder during which histamine released into the circulation causes vasodilation as well as increased permeability of the capillaries with resultant marked loss of plasma from the circulation. Anaphylaxis can occur systemically, with the associated effects experienced over the entire body, and it can occur locally, with the reaction limited to a specific target tissue or organ.

[0074] The term "viral disease", as used herein, refers to a disease which has a virus as its etiologic agent. Examples of viral diseases include hepatitis B, hepatitis C, influenza, acquired immunodeficiency syndrome (AIDS), and herpes zoster.

[0075] As used herein, and as well-understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the

disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

**[0076]** "Palliating" a disease or disorder means that the extent and/or undesirable clinical manifestations of a disorder or a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not treating the disorder. Especially in the allergy context, as is well-understood by those skilled in the art, palliation may occur upon modulation of the immune response against an allergen(s). Further, palliation does not necessarily occur by administration of one dose, but often occurs upon administration of a series of doses. Thus, an amount sufficient to palliate a response or disorder may be administered in one or more administrations.

**[0077]** An "antibody titer", or "amount of antibody", which is "elicited" by a CIC and antigen refers to the amount of a given antibody measured at a time point after administration of the CIC and antigen.

**[0078]** A "Th1-associated antibody" is an antibody whose production and/or increase is associated with a Th1 immune response. For example, IgG2a is a Th1-associated antibody in mouse. For purposes of this invention, measurement of a Th1-associated antibody can be measurement of one or more such antibodies. For example, in humans, measurement of a Th1-associated antibody could entail measurement of IgG1 and/or IgG3.

**[0079]** A "Th2-associated antibody" is an antibody whose production and/or increase is associated with a Th2 immune response. For example, IgG1 is a Th2-associated antibody in mouse. For purposes of this invention, measurement of a Th2-associated antibody can be measurement of one or more such antibodies. For example, in human, measurement of a Th2-associated antibody could entail measurement of IgG2 and/or IgG4.

**[0080]** To "suppress" or "inhibit" a function or activity, such as cytokine production, antibody production, or histamine release, is to reduce the function or

activity when compared to otherwise same conditions except for a condition or parameter of interest, or alternatively, as compared to another condition. For example, a composition comprising a CIC and antigen which suppresses histamine release reduces histamine release as compared to, for example, histamine release induced by antigen alone. As another example, a composition comprising a CIC and antigen which suppresses antibody production reduces extent and/or levels of antibody as compared to, for example, extent and/or levels of antibody produced by antigen alone.

[0081] As used herein manufactured or formulated "under GMP standards," when referring to a pharmaceutical composition means the composition is formulated as sterile, substantially isotonic and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

[0082] As used herein, the term "immunogenic" has the normal meaning in the art and refers to an agent (*e.g.*, polypeptide) that elicits an adaptive immune response upon injection into a person or animal. The immune response may be B cell (humoral) and/or T cell (cellular).

[0083] All ranges are intended to be inclusive of the terminal values. Thus, a polymer of "from 2 to 7 nucleotides" or "between 2 and 7 nucleotides" includes polymers of 2 nucleotides and polymers of 7 nucleotides. Where a lower limit and an independently selected upper limit are described, it is understood that the upper limit is higher than the lower limit.

### III. Chimeric Immunomodulatory Compounds

[0084] The invention provides chimeric immunomodulatory compounds ("CICs") useful, *inter alia*, for modulating an immune response in individuals such as mammals, including humans. The present invention is based, in part, on the discovery that some chimeric molecules containing nucleic acid moieties covalently bound to non-nucleic acid spacer moieties have immunomodulatory activity,

particularly in human cells. Surprisingly, this activity is manifest even in cases in which the nucleic acid moieties have a sequence that, if presented as an isolated polynucleotide, do not exhibit significant immunomodulatory activity.

**[0085]** Thus, the invention provides new reagents and methods for modulating an immune response, including treatment and prophylaxis of disease in humans and other animals.

**[0086]** The following sections describe the structure and properties of the CICs of the invention, as well as the structure and properties of the component nucleic acid moieties and non-nucleic acid spacer moieties.

#### 1. Core Structure of CIC

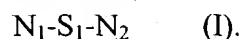
**[0087]** CICs of the present invention contain one or more nucleic acid moieties and one or more non-nucleic acid spacer moieties. CICs having a variety of structures are contemplated. For illustration, exemplary CICs have core structures described in formulas I-VIII, below. Formulas I-III show core sequences for "linear CICs." Formulas IV-VI show core sequences for "branched CICs." Formula VIII shows a core structure for "single-spacer CICs."

**[0088]** In each formula provided below, "N" designates a nucleic acid moiety (oriented in either a 5'→3' or 3'→5' orientation) and "S" designates a non-nucleic acid spacer moiety. A dash ("-") designates a covalent bond between a nucleic acid moiety and a non-nucleic acid spacer moiety. A double dash ("--") designates covalent bonds between a non-nucleic acid spacer moiety and at least 2 nucleic acid moieties. A triple dash ("---") designates covalent bonds between a non-nucleic acid spacer moiety and multiple (i.e., at least 3) nucleic acid moieties. Subscripts are used to designate differently positioned nucleic acid or non-nucleic acid spacer moieties. However, the use of subscripts to distinguish different nucleic acid moieties is not intended to indicate that the moieties necessarily have a different structure or sequence. Similarly, the use of subscripts to distinguish different spacer moieties is

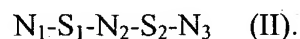
not intended to indicate that the moieties necessarily have different structures. For example, in formula II, *infra*, the nucleic acid moieties designated N<sub>1</sub> and N<sub>2</sub> can have the same or different sequences, and the spacer moieties designated S<sub>1</sub> and S<sub>2</sub> can have the same or different structures.

#### A. Linear CICs

[0089] In one embodiment, the CIC comprises the core structure



[0090] In one embodiment, the CIC comprises the core structure



[0091] In one embodiment, the CIC comprises the core structure



where A is an integer between 1 and about 100 and [N<sub>v</sub>-S<sub>v</sub>] indicates A additional iterations of nucleic acid moieties conjugated to non-nucleic acid spacer moieties.

The subscript "v" indicates that N and S are independently selected in each iteration of "[N<sub>v</sub>-S<sub>v</sub>]." "A" is sometimes between 1 and about 10, sometimes between 1 and 3, sometimes exactly 1, 2, 3, 4 or 5. In some embodiments, A is an integer in a range defined by a lower limit of 1, 2, 3, 4, or 5, and an independently selected upper limit of 10, 20, 50 or 100 (*e.g.*, between 3 and 10). A non-nucleic acid spacer moiety that is covalently linked to exactly two nucleic acid moieties (*e.g.*, S<sub>1</sub> in structures I-III, *supra*) can be referred to as a "linear spacer."

[0092] In some embodiments of the invention, the CIC has the structure of formula I, II or III. However, according to the invention, in some embodiments, linear CICs comprise, but are not necessarily limited to, the structures provided in formulas I-III. That is, formulas I, II, and III define core structures in which the non-nucleic acid spacer moieties in the core structure are covalently bound to no more than two nucleic acid moieties. However, it is contemplated that, in many embodiments, additional chemical moieties (*e.g.*, phosphate, mononucleotide,

additional nucleic acid moieties, alkyl, amino, thio or disulfide groups or linking groups, and/or spacer moieties) are covalently bound at the termini of the core structures. For example, if all nucleic acid moieties in a CIC are 5'-TCGTCGA-3', and spacer moieties are selected from hexaethylene glycol ("HEG"), a phosphorothioate-linked multimer of HEG, and glycerol, CICs having a core structure of formula I include each of the following formulas:

TCGTCGA-HEG-TCGTCGA-OH	(Ia)
TCGTCGA-HEG-TCGTCGA-PO <sub>4</sub>	(Ib)
TCGTCGA-HEG-TCGTCGA-HEG	(Ic)
HEG-TCGTCGA-HEG-TCGTCGA-HEG	(Id)
TCGTCGA-HEG-TCGTCGA-HEG-TCGTCGA	(Ie)
TCGTCGA-HEG-TCGTCGA-(HEG) <sub>4</sub> -TCGTCGA	(If)
(TCGTCGA) <sub>2</sub> -glycerol-TCGTCGA-HEG-TCGTCGA	(Ig)
PO <sub>4</sub> -TCGTCGA-HEG-TCGTCGA	(Ih)
TCGTCGA-(HEG) <sub>15</sub> -T	(Ii)
(TCGTCGA-HEG) <sub>2</sub> -glycerol-HEG-TCGTCGA	(Ij)
TCGTCGA-HEG-T-HEG-T	(Ik)

**[0093]** It will be immediately apparent that the genus of CICs comprising a core structure of formula I encompasses CICs comprising a core structure of formula II or III.

**[0094]** In some embodiments, one or more spacers comprises smaller units (*e.g.*, oligoethylene glycols [*e.g.*, HO-(CH<sub>2</sub>CH<sub>2</sub>-O)<sub>N</sub>-H, where N = 2-10, such as HEG and TEG], glycerol, C3 alkyl, and the like) linked together. In one embodiment, the linkage is an ester linkage (*e.g.*, phosphodiester or phosphorothioate ester) or other linkage, *e.g.*, as described *infra*.

[0095] In certain embodiments, the terminal structures of the CIC are covalently joined (*e.g.*, nucleic acid moiety-to-nucleic acid moiety; spacer moiety-to-spacer moiety, or nucleic acid moiety-to-spacer moiety), resulting in a circular conformation.

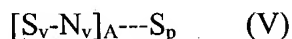
#### B. Branched CICs

[0096] In one embodiment, the CIC comprises the core structure

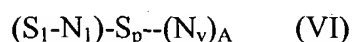


where  $S_p$  is a multivalent spacer covalently bonded to the quantity "A" independently selected nucleic acid moieties  $N_v$ , and where A is at least 3, *e.g.*, exactly 3, 4, 5, 6, or 7 or more than 7. In various embodiments, A is an integer between 3 and 100 (inclusive). In some embodiments, A is an integer in a range defined by a lower limit of about 3, 5, 10, 50, or 100 and an independently selected upper limit of about 5, 7, 10, 50, 100, 150, 200, 250, or 500. It is also contemplated that in some embodiments, A is greater than about 500.

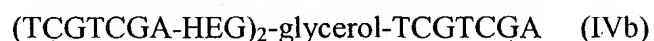
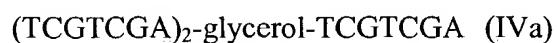
[0097] In a related embodiment, the CIC comprises the core structure



where  $S_p$  is a multivalent spacer covalently bonded to the quantity "A" independently selected elements,  $S_v-N_v$ , comprising a spacer moiety covalently bound to a nucleic acid moiety, and where A is at least 3. In various embodiments, A is an integer between 3 and 100 (inclusive). In some embodiments, A is an integer in a range defined by a lower limit of 5, 10, 50, or 100 and an independently selected upper limit of 10, 50, 100, 250, or 500. It is also contemplated that in some embodiments, A is greater than 500. In a related embodiment, the CIC comprises the core structure:



where  $S_p$  is a multivalent spacer covalently bonded to the quantity "A" independently selected nucleic acid moieties,  $N_v$ , and at least one nucleic acid moiety  $N_1$  bound to a spacer moiety  $S_1$ , where A is at least 2. In one embodiment, A is 2. In various embodiments, A is 3, is 4, is 5, or is an integer between 3 and 100 (inclusive). In some embodiments, A is an integer in a range defined by a lower limit of 5, 10, 50, or 100 and an independently selected upper limit of 10, 50, 100, 150, 200, 250, or 500. It is also contemplated that in some embodiments, A is greater than 500. In some embodiments of the invention, the CIC has the structure of formula I, II or III. However, according to the invention, branched CICs comprise, but are not limited to, the structures provided in formulas IV, V and VI. That is, formulas IV, V and VI define core structures in which a multivalent spacer moiety ( $S_p$ ) is covalently bound to at least three (3) nucleic acid moieties. It is contemplated that, in some embodiments, additional chemical moieties (*e.g.*, phosphate, mononucleotide, additional nucleic acid and/or spacer moieties) are covalently bound at the termini of the core structures. For example, if all nucleic acid moieties in a CIC are 5'TCGTCGA 3' and all spacer moieties are glycerol or HEG, CICs having a core structure of formula IV include:



[0098] It will be immediately apparent, for example, that the genus of CICs comprising a core structure of formula IV encompasses CICs comprising a core structure of formula V or VI. In a preferred embodiment of the invention, the CIC comprises at least two different (*i.e.*, different sequence) nucleic acid moieties.



[0099] In some embodiments, one or more spacers comprises smaller units (e.g., HEG, TEG, glycerol, C3 alkyl, and the like) linked together. In one embodiment, the linkage is an ester linkage (e.g., phosphodiester or phosphorothioate ester).

[0100] A non-nucleic acid spacer moiety that is covalently linked to more than two nucleic acid moieties can be referred to as a "multivalent spacer." As is discussed below, examples of multivalent spacers include glycerol, FICOLL<sup>®</sup>, and dendrimer moieties that are covalently linked to more than two nucleic acid moieties. (Glycerol, for example, can also be a linear spacer, if it is linked to only two nucleic acid moieties; see Example 11.)

[0101] For convenience, a multivalent spacer with a low valency is sometimes called a "branched spacer" or "branching spacer." A multivalent spacer with low valency is a multivalent spacer that is readily covalently linked to not more than 10 nucleic acid moieties, usually fewer than 6, sometimes fewer than 4 and sometimes 3 nucleic acid moieties often or, ) Examples of multivalent spacer with a low valency include glycerol, 1,3-diamino-2-propanol and substituted derivatives (e.g., "symmetrical doubler"), pentaerithritol derivatives (e.g., "trebler"), and the like. In contrast, multivalent spacers can readily covalently bind >10 nucleic acid moieties, and are often are capable of covalent linkage to >50, >100 or >200 nucleic acid moieties. Examples of multivalent spacer with a high valency include Ficoll<sup>®</sup>, dextran, and other modified polysaccharides, "Starburst<sup>®</sup> dendrimers of Generation 2-5 (valency 16-128), and the like.

### C. CICs Having Specified Tertiary Structure, and CIC Multimers

[0102] The linear and branched CICs described herein (e.g., in Sections B and C, *supra*) include variants having particular structural features. CICs and CIC multimers described in this section may be targeted to, or efficiently taken up by phagocytic cells or antigen-presenting cells, may present a high density of nucleic acid moiety 5'-ends, may change structure *in vivo* (e.g., due to nuclease or other degradative activity,

acidification in the endosome, and/or dilution of the CIC or multimer *in vivo* (thereby changing properties after administration to a subject or in a particular biological compartment).

i) CICs having specified tertiary structure

[0103] As noted elsewhere herein, linear CICs with at least two nucleic acid moieties having sequences complementary or partially complementary to each other can form hairpin duplexes (and/or CIC dimers or concatamers, as discussed below). As used herein, "hairpin duplex" refers to the structure formed by hybridization of two nucleic acid moieties that are in the same orientation in the CIC (e.g., one nucleic acid moiety is bound at the 3' terminus to the spacer moiety and the other nucleic acid moiety is bound at the 5' terminus to the spacer moiety) in a CIC. In one embodiment, the two nucleic acid moieties are separated by no more than one additional nucleic acid moiety. In another embodiment, there is no intervening nucleic acid moiety between the base-paired nucleic acid moieties. Examples of CICs that may form hairpin duplexes, provided for illustration and not limitation, are C-159 and C-160 shown *infra* in Table 2 and the Examples). Also see Figure 8A. In a hairpin duplex, the pair of nucleic acid moieties with complementary sequences can be self-complementary (e.g., palindromic) or the pair can have different sequences. It will be appreciated that exact complementarity is not required so long as the nucleic acid moieties are of sufficient complementarity and length to form a duplex at 37°C in an aqueous solution at physiological pH (i.e., 7.0-7.4, e.g., 7.2) and ionic strength (e.g., 150 mM NaCl).

[0104] The presence of a duplex structure can be detected using well-known methods. These include detecting a change in CIC structure based on size exclusion chromatography, and detecting a change in  $A_{260}$  or  $A_{280}$  upon raising or lowering the temperature of the CIC-containing composition (indicative of melting or formation of

the duplex). Absorbance increases as a double-stranded DNA separates into the single-stranded forms.

**[0105]** As noted, certain CICs can form hairpin structures or can form dimers or concatamers. It is believed the latter structures are favored when the CICs are allowed to anneal at high concentration and/or when the spacer is of sufficient length and flexibility (e.g., [HEG]<sub>6</sub>) to favor the kinetics of dimer formation by providing increased degrees of freedom of movement of the nucleic acid moieties.

**[0106]** Like linear CICs, branched CICs can form a variety of types of structures, including the "fork," "H," "comb," "central spacer," and "dendrimer" structures described below and in the Examples.

**[0107]** A "fork" structure has only a single branching spacer (e.g. glycerol, glycerol-[HEG]<sub>2</sub>, symmetrical doubler-[HEG]<sub>2</sub>, and the like), which is bound to three nucleic acid moieties, as illustrated in Figure 8B (CIC C-155; C-35). The three nucleic acid moieties can all have the same sequence, or can have different sequences. In one embodiment, at least 2 of the nucleic acid moieties has the same sequence. In one embodiment, at least 1, at least 2, or at least 3 of the nucleic acid moieties is a 5-prime moiety (see § 3(C), *infra* for an explanation of this nomenclature). In an embodiment, at least 1, at least 2, or at least 3 of the nucleic acid moieties includes the sequence CG, optionally TCG, optionally 5'<sup>F</sup>-TCG (i.e., TCG in the 5-prime position of a 5-prime moiety; see § 3(C), *infra* for an explanation of this nomenclature). The reader will recognize that one or more of the nucleic acid moieties can have a sequence, motif or property described herein below (e.g., § III(2)-(3)).

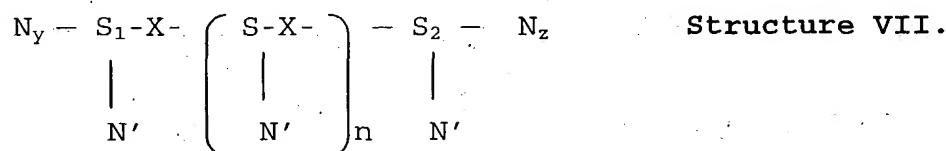
**[0108]** A "trident" structure has only a single branching spacer (e.g., trebler, [HEG]-trebler-[HEG]<sub>3</sub>, and the like), which is bound to four nucleic acid moieties. The four nucleic acid moieties can all have the same sequence, or can have different sequences. In one embodiment, at least 3 of the nucleic acid moieties has the same sequence. In one embodiment, at least 1, at least 2, at least 3, or at least 4 of the

nucleic acid moieties is a 5-prime moiety. In an embodiment, at least 1, at least 2, at least 3, or at least 4 of the nucleic acid moieties includes the sequence CG, optionally TCG, optionally 5'<sup>F</sup>-TCG. The reader will recognize that one or more of the nucleic acid moieties can have a sequence, motif or property described herein below (e.g., § III(2)-(3)).

**[0109]** A “polydent” structure has at least 3 branched spacers (e.g., 3-15, usually 3-7) and at least 4 nucleic acid moieties, where all of the nucleic acid moieties in the structure have an unbound terminus (a free 5' end or a free 3' end). In one embodiment all of the nucleic acid moieties have a free 5'-end. See, e.g., C-126 (Table 2).

**[0110]** An “H” structure is defined by having exactly two branching spacers, each of which is linked to the other via (a) a nucleic acid moiety or (b) a combination of nucleic acid moieties and nonbranching spacers (e.g., -ATTT-HEG-ATTT-) and each of which is linked to two additional nucleic acid moieties. An example is CIC C-171 (see Figure 8C). In embodiments, at least 1, at least 2, at least 3 or at least 4 (i.e., all) of the “two additional nucleic acid moieties” is a 5-prime moiety. In one embodiment, at least 1, at least 2, at least 3, or at least 4 of the two additional nucleic acid moieties is a 5-prime moiety. In embodiments, at least 1, at least 2, at least 3, or at least 4 of the nucleic acid moieties includes the sequence CG, optionally TCG, optionally 5'<sup>F</sup>-TCG. The reader will recognize that one or more of the nucleic acid moieties can have a sequence, motif or property described herein below (e.g., § III(2)-(3)). The nucleic acid moiety(s) linking the two branching spacers may also comprise a sequence CG or other sequence or motif described herein.

**[0111]** A “comb” structure comprises the following structure VII:



wherein y and z are independently 0 or 1, and n can be from 1 to 10, preferably 3 to 6, most preferably 3 or 4. In this formula, each S (including  $S_1$  and  $S_2$ ) represents a spacer (which may be the same or different), and is always branched spacer, unless x or y is 0, in which case the end spacer(s) [represented by  $S_1$  and  $S_2$ ] will be linear.  $N'$ ,  $N_y$  and  $N_z$  are nucleic acid moieties where each  $N'$  has the same sequence. Each X represents the structure  $[(N-LS)_m - N]$ , where m is from 0 to 5, usually 0 or 1; where each N (as well as  $N_y$  and  $N_z$ ) is independently selected and represents a nucleic acid moiety which may be the same or different; and where each LS represents a linear spacer, where each linear spacer is independently selected and may be the same or different. In various embodiments, at least one nucleic acid moiety is a 5-prime moiety and/or includes the sequence CG, optionally TCG, optionally 5'-TCG. In an embodiment, each  $N'$  is a 5-prime moiety. In one embodiment all of the 5-prime moieties have the same sequence and/or all of the nucleic acid moieties that are not 5' moieties have the same sequence. The reader will recognize that one or more of the nucleic acid moieties can have a sequence, motif or property described hereinbelow (e.g., § III(2)-(3)). An example of a comb structure is C-169 (see Figure 8D). In comb structures, the branched spacers may be the same. Alternatively, a comb structure may contain 2 or more different branched spacers.

**[0112]** A "central spacer" structure is defined by having spacer moiety bound to 4 or more nucleic acid moieties, where at least 3 of said 4 or more nucleic acid moieties is a 5-prime moiety, and wherein at least 3 of the 5-prime moieties include the sequence CG, optionally TCG, optionally 5'-TCG. The reader will recognize that one or more of the nucleic acid moieties can have a sequence, motif or property described hereinbelow (e.g., § III(2)-(3)). See C-139, C-140, C-168, C-170, and Figures 8E, 8F, and 8G. In various embodiments, the number of nucleic acid moieties bound to the spacer may be less than 500 (e.g., for CICs made by conjugation strategies, such as CICs with Ficoll-based central spacers) or less than about 10 (e.g., for compounds made using a DNA synthesizer, e.g C-168 and C-170).

[0113] A “CIC dendrimer” is a discrete, highly branched polymer created by covalent linking of multiple (e.g., 3-15) branched CICs. Usually all or most of the component CICs has the same structural motif (e.g., all are fork structures or all are trident structures). For example, Figure 8H shows a third generation CIC dendrimer produced by linking 7 fork structure CICs. Also see structure IVd in Section § III(1)(b), an example of a 2<sup>nd</sup> generation dendrimer containing 3 fork CICs. The CIC dendrimer should not be confused with dendrimers that may serve as spacer moieties but which do not comprise nucleic acid moieties (e.g., the “dense star or “starburst” polymers described hereinbelow).

ii) CIC Multimers

[0114] Certain CIC linear or branched CICs of the invention can form “multimers” of 2 or more CICs that stably associate with each other due to Watson-Crick hybridization between pairs of at least partially complementary nucleic acid moieties. Examples of such CIC multimers are multimers comprising only linear CICs (e.g., see Figures 9A-D) and CIC multimers comprising at least one, and usually at least two, branched CICs (e.g., see Figures 9E-G).

[0115] Examples of CIC multimers comprising only linear CICs include dimers shown in Figure 9A (showing dimers where the component linear CICs are the same), Figure 9B (showing dimers where the component linear CICs are different), Figure 9C (showing dimers having 5' ends that are not basepaired), and Figure 9D (showing a concatamer of linear CICs). Examples of CIC multimers comprising branched CICs are shown in Figure 9E (showing a “central axis” structure), Figure 9F (showing a “cage” structure), and Figure 9G (showing a “starfish” structure). It will be understood the multimers of Figure 9 are provided for illustration and not limitation. Thus, the majority of CIC multimers shown in Figure 9 are assemblies of two CICs. In various alternative embodiments CIC multimers may comprise at least 2, at least 3, at least 4, at least 5, at least 10, and sometimes more than 10 individual CICs. The individual CIC subunits need not all be the same.

[0116] As noted, individual CICs in CIC multimers stably associate with each other. As used in this context, "stably associate" means the CICs remain associated at 37°C in a buffered aqueous salt solution of near physiological ionic strength and pH, e.g., 150 mM NaCl, pH 7.2. It will be recognized, of course, that even "stably associated" multimeric macromolecules may exist in a state of equilibrium such that an individual CICs may be unassociated with the multimer for relatively brief periods of time, or there may be exchange between CICs in the multimeric structure and unassociated monomers in solution. CIC multimers may be self assembling (i.e., the component CICs may spontaneously associate under physiological conditions). Usually, a CIC multimer will form when the component CICs are dissolved at a concentration of approximately 1.0 mg/ml in 50 mM sodium phosphate/150 mM sodium chloride/pH 7.2, heated to 95°C for 3 min., and allowed to slowly (e.g., over a period of approximately 2 hours) to 37°C or room temperature. See Example 51, *infra*.

[0117] Because the association between CICs in a CIC multimer relies, at least in part, on hybrids formed between nucleic acid moieties that are at least partially complementary, and sometimes exactly complementary, the normal parameters for formation of nucleic acid hybrids apply. That is, the hybridizing regions of nucleic acid moieties are of sufficient length and/or sequence composition (e.g., GC content) to form stable CIC multimers. Generally the nucleic acid moieties of one CIC will comprise at least 8, more often at least 10, and usually at least 12 contiguous bases that are exactly complementary to nucleic acid moieties of a second CIC in the multimer. However, when there are a large number of hybridizing nucleic acid moieties, the region of complementarity or contiguity may be shorter.

[0118] Conditions under which two polynucleotides, or regions of a self-complementary polynucleotide, will form a duplex can be determined empirically or can be predicted using are well known methods (taking into consideration base sequence, polynucleotide length, type of ester linkage [e.g., phosphorothioate or

phosphodiester linkage], temperature, ionic strength, presence of modified bases or sugars, etc.). The annealing nucleic acid moieties in the associating CICs may be selfcomplementary (see, e.g., Figure 9F) or alternatively, a nucleic acid moiety(s) on one CIC may be complementary to a nucleic acid moiety(s) on a second CIC, but not to itself.

**[0119]** As noted above, examples of CIC multimers include multimers having a "central axis" structure, a "cage" structure, and a "starfish" structure." A "central axis" structure refers to a dimer of two branched CICs, in which one nucleic acid moiety of each CIC forms a double-stranded region with a complementary nucleic acid moiety of the second CIC, and each spacer is bound to at least two other nucleic acid moieties. See Figure 9E.

**[0120]** A "cage" structure refers to a CIC multimer in which at least two nucleic acid 5-prime moieties from each component CIC are hybridized to a nucleic acid moiety of another CIC in the multimer. See Figure 9F. In some embodiments, all of the 5-prime moieties from one or all of the CICs are hybridized to a nucleic acid moiety of another CIC in the multimer. A "cage" structure is characterized in that each of the nucleic acid 5-prime moieties in a duplex is linked to the spacer moiety with the same polarity (i.e., the spacer moiety-nucleic acid moiety linkage for each nucleic acid moiety in a particular duplex is either 3' or is 5'). In an embodiment, the cage structure CIC multimer contains no more than two CICs.

**[0121]** A "starfish" structure has the same properties as the cage structure, *supra*, except (a) the starfish is always a dimer and (b) the two nucleic acid moieties in each duplex are linked to the spacer moieties with different polarities (i.e., one is linked at the 5' terminus and one is linked at the 3' terminus). See Figure 9G.

**[0122]** In each type of CIC multimer, it will be understood that nucleic acid moieties in the multimer may have any of the sequence, structural features or properties described herein for nucleic acid moieties, so long as the feature is consistent with the multimer structure. Thus, one or more nucleic acid moieties may



be a 5-prime moiety, may include the sequence CG, TCG, or 5'<sup>F</sup>-TCG, or have other sequence, motif or property described herein (e.g., § III(2)-(3)). Further, it will be understood the multimers of Figure 9 are provided for illustration and not limitation.

[0123] Examples 58 and 59 illustrate that tertiary structure and multimerization can enhance the activity of CICs. The results of Example 58 show that CICs that can self-hybridize (C-173, C-174, C-175) induced significantly more IFN- $\alpha$  from human PBMC than did the parent oligonucleotide (P-17) when used at low doses (e.g., 0.8 ug/ml). The results of Example 59 show that CICs that hybridize to produce a total of four free 5'-ends with active TCG-containing heptamers (e.g., C-C178 duplex, C-202/C-203 heteroduplex) induced significantly more IFN- $\alpha$  from human PBMC than CICs containing only two free 5'-ends (C-101, C-202, C-203).

#### D. Single-Spacer CICs

[0124] In a different aspect of the invention, the CIC comprises a structure in which there is a single nucleic acid moiety covalently conjugated to a single spacer moiety, i.e.,



[0125] In one embodiment,  $S_1$  has the structure of a multimer comprising smaller units (e.g., oligoethylene glycols, [e.g., HO-(CH<sub>2</sub>CH<sub>2</sub>-O)<sub>N</sub>-H, where N = 2-10; e.g., HEG and TEG], glycerol, 1'2'-dideoxyribose, C2 alkyl - C12 alkyl subunits [preferably, C2 alkyl - C10 alkyl subunits], and the like), typically connected by an ester linkage (e.g., phosphodiester or phosphorothioate ester), e.g., as described *infra*. See, e.g., formula VIIa, *infra*. The multimer can be heteromeric or homomeric. In one embodiment, the spacer is a heteromer of monomeric units (e.g., HEG, TEG, glycerol, 1'2'-dideoxyribose, C2 alkyl to C12 alkyl linkers, preferably C2 alkyl to C10 alkyl linkers, and the like) linked by an ester linkage (e.g., phosphodiester or phosphorothioate ester). See, e.g., formula VIIb, *infra*.

[0126] For example, if the nucleic acid moiety is 5'TCGTCGA 3' and the spacer moiety is a phosphorothioate-linked multimer of hexaethylene glycol ["(HEG)<sub>15</sub>"], a CIC having a core structure of formula VII includes:



[0127] Similarly, if the nucleic acid moiety is 5'TCGTCGA 3' and the spacer moiety is a phosphorothioate-linked multimer of alternating hexaethylene glycol and propyl subunits, a CIC having a core structure of formula VI includes:



## 2. Immunomodulatory Activity of CICs

[0128] The CICs of the invention have immunomodulatory activity. The terms "immunomodulatory," "immunomodulatory activity," or "modulating an immune response," as used herein, include immunostimulatory as well as immunosuppressive effects. An immune response that is immunomodulated according to the present invention is generally one that is shifted towards a "Th1-type" immune response, as opposed to a "Th2-type" immune response. Th1-type responses are typically considered cellular immune system (*e.g.*, cytotoxic lymphocytes) responses, while Th2-type responses are generally "humoral", or antibody-based. Th1-type immune responses are normally characterized by "delayed-type hypersensitivity" reactions to an antigen. Th1-type responses can be detected at the biochemical level by increased levels of Th1-associated cytokines such as IFN- $\gamma$ , IFN- $\alpha$ , IL-2, IL-12, and TNF- $\alpha$ , as well as IL-6, although IL-6 may also be associated with Th2-type responses as well. Th2-type immune responses are generally associated with higher levels of antibody production, including IgE production, an absence of or minimal CTL production, as well as expression of Th2-associated cytokines such as IL-4 and IL-5.

[0129] Immunomodulation in accordance with the invention may be recognized by measurements (assays) *in vitro*, *in vivo* and/or *ex vivo*. Examples of measurable immune responses indicative of immunomodulatory activity include, but are not limited to, antigen-specific antibody production, secretion of cytokines, activation or expansion of lymphocyte populations such as NK cells, CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, B lymphocytes, and the like. See, e.g., WO 97/28259; WO 98/16247; WO 99/11275; Krieg et al. (1995) *Nature* 374:546-549; Yamamoto et al. (1992) *J. Immunol.* 148:4072-4076; Ballas et al. (1996) *J. Immunol.* 157:1840-1845; Klinman et al. (1997) *J. Immunol.* 158:3635-3639; Sato et al. (1996) *Science* 273:352-354; Pisetsky (1996) *J. Immunol.* 156:421-423; Shimada et al. (1986) *Jpn. J. Cancer Res.* 77:808-816; Cowdery et al. (1996) *J. Immunol.* 156:4570-4575; Roman et al. (1997) *Nat Med.* 3:849-54; Lipford et al. (1997) *Eur. J. Immunol.* 27:2340-2344; WO 98/55495, WO 00/61151, Pichyangkul et al. (2001) *J. Imm. Methods* 247:83-94. See also the Examples, *infra*. Certain useful assays are described herein below for purposes of illustration and not for limitation.

[0130] Assays are generally carried out by administering or contacting a cell, tissue, animal or the like with a test sample (e.g., containing a CIC, polynucleotide, and/or other agent) and measuring a response. The test samples containing CICs or polynucleotides can be in a variety of forms or concentrations, which will be understood by the ordinarily skilled practitioner to be appropriate for the assay type. For example, for purposes of a cell-based assay, CICs or polynucleotides are often used at a concentration of 20 µg/ml or 10 µg/ml or 2 µg/ml. Typically, for the purposes of the assay, concentration is determined by measuring absorbance at 260 nm and using the conversion  $0.5 \text{ OD}_{260}/\text{ml} = 20 \text{ µg/ml}$ . This normalizes the amount of total nucleic acid in the test sample and may be used, for example, when the spacer moiety does not have a significant absorbance at 260 nm. Alternatively, concentration or weight can be measured by other methods known in the art. If desired, the amount of nucleic acid moiety can be determined by measuring

absorbance at 260 nm, and the weight of the CIC calculated using the molecular formula of the CIC. This method is sometimes used when the ratio of weight contributed by the spacer moiety(s) to weight contributed by the nucleic acid moieties in a CIC is high (i.e., greater than 1).

[0131] It will similarly be understood that positive and negative controls are useful in assays for immunomodulatory activity. A suitable positive control for immunomodulatory activity is the immunomodulatory phosphorothioate DNA having the sequence 5'-TGACTGTGAACGTTTCGAGATGA-3' (SEQ ID NO:2), although other suitable positive controls with immunomodulatory activity will be apparent to the ordinarily skilled practitioner. One suitable negative control is no test agent (i.e., excipient or media alone, also referred to as "cells alone" for certain *in vitro* assays). Alternatively, a phosphorothioate DNA having the sequence 5'-TGACTGTGAACCTTAGAGATGA-3' (SEQ ID NO:3) is used as a negative control in some embodiments. Other negative controls can be designed by the practitioner guided by the disclosure herein and ordinary assay design.

[0132] One useful class of assays is "cytokine response assays." An exemplary assay for immunomodulatory activity measures the cytokine response of human peripheral blood mononuclear cells ("PBMCs") (e.g., as described in Bohle et al. [1999], *Eur. J. Immunol.* 29:2344-53; Verthelyi et al. [2001] *J. Immunol.* 166:2372-77). In one embodiment of this assay, peripheral blood is collected from one or more healthy human volunteers and PBMCs are isolated. Typically blood is collected by venipuncture using a heparinized syringe, layered onto a FICOLL<sup>®</sup> (Amersham Pharmacia Biotech) cushion and centrifuged. PBMCs are then collected from the FICOLL<sup>®</sup> interface and washed twice with cold phosphate buffered saline (PBS). The cells are resuspended and cultured (e.g., in 48- or 96-well plates) at  $2 \times 10^6$  cells/mL in RPMI 1640 with 10% heat-inactivated human AB serum, 50 units/mL penicillin, 50 µg/mL streptomycin, 300 µg/mL glutamine, 1 mM sodium pyruvate,

and 1 x MEM non-essential amino acids (NEAA) in the presence and absence of test samples or controls for 24 hours.

**[0133]** Cell-free medium is collected from each well and assayed for IFN- $\gamma$  and/or IFN- $\alpha$  concentration. Immunomodulatory activity is detected when the amount of IFN- $\gamma$  secreted by PBMCs contacted with the test compound is significantly greater (*e.g.*, at least about 3-fold greater, usually at least about 5-fold greater) than the amount secreted by the PBMCs in the absence of the test compound or, in some embodiments, in the presence of an inactive control compound (*e.g.*, 5'-TGACTGTGAACCTTAGAGATGA-3' (SEQ ID NO:3)). Conversely, a test compound does not have immunomodulatory activity if the amount of IFN- $\gamma$  secreted by PBMCs contacted with the test compound is not significantly greater (*e.g.*, less than 2-fold greater) than in the absence of the test compound or, alternatively, in the presence of an inactive control compound (*e.g.*, 5'-TGACTGTGAACCTTAGAGATGA-3' (SEQ ID NO:3)).

**[0134]** When IFN- $\alpha$  concentration is assayed, the amount of IFN- $\alpha$  secreted by PBMCs contacted with the test compound is often significantly greater (*e.g.*, in the case of IFN- $\alpha$  sometimes at least about 2-fold or at least about 3-fold greater) than the amount secreted by the PBMCs in the absence of the test compound or, in some embodiments, in the presence of an inactive control compound (*e.g.*, 5'-TGACTGTGAACCTTAGAGATGA-3' (SEQ ID NO:3)). In some embodiments, the significantly increased IFN- $\alpha$  secretion level is at least about 5-fold, at least about 10-fold, or even at least about 20-fold greater than controls. Conversely, a test compound does not have immunomodulatory activity if the amount of IFN- $\alpha$  secreted by PBMCs contacted with the test compound is not significantly greater (*e.g.*, less than 2-fold greater) than in the absence of the test compound or, alternatively, in the presence of an inactive control compound (*e.g.*, 5'-TGACTGTGAACCTTAGAGATGA-3' (SEQ ID NO:3)).

[0135] As illustrated in the examples, *infra*, administration of some CICs results in significant secretion of both IFN- $\gamma$  and IFN- $\alpha$ , while administration of other CICs has a lesser effect on secretion of IFN- $\alpha$  or, conversely, a lesser effect on secretion of IFN- $\gamma$ . See, e.g., Example 49.

[0136] Another useful class of assays are cell proliferation assays, e.g., B cell proliferation assays. The effect of an agent (e.g. a CIC) on B cell proliferation can be determined using any of a variety of assays known in the art. An exemplary B cell proliferation assay is provided in Example 41.

[0137] To account for donor variation, e.g., in cell-based assays, such as cytokine and proliferation assays, preferably assays are carried out using cells (e.g., PBMCs) from multiple different donors. The number of donors is usually at least 2 (e.g. 2), preferably at least 4 (e.g. 4), sometimes at least 10 (e.g. 10). Immunomodulatory activity is detected when the amount of IFN- $\gamma$  secreted in the presence of the test compound (e.g. in at least half of the healthy donors tested, preferably in at least 75%, most preferably in at least 85%) is at least about 3-fold greater or at least about 5-fold greater than secreted in the absence of the test compound, or in some embodiments, than in the presence of an inactive control compound such as described *supra*.

[0138] Immunomodulatory activity may also be detected by measuring interferon-induced changes in expression of cytokines, chemokines and other genes in mammalian cells (e.g., PBMCs, bronchial alveolar lavage (BAL) cells, and other cells responsive to interferon). For example, expression of the chemokines interferon-induced-protein 10kDa (IP-10), monokine induced by IFN- $\gamma$  (MIG) and monocyte chemotactic protein 1 (MCP-1) are increased in the presence of IFN- $\alpha$  and IFN- $\gamma$ . Expression of these proteins, or their corresponding mRNA, may be used as markers of immunostimulatory activity in cultured cells or tissues or blood of animals to which a CIC has been administered. Expression of such markers can be monitored

any of a variety of methods of assessing gene expression, including measurement of mRNAs (e.g., by quantitative PCR), immunoassay (e.g., ELISA), and the like.

[0139] Biological activity of CICs can also be measured by measuring the induction of gene products known to have antiviral activities, including 2'-5' Oligoadenylate synthetase (2'-5'OAS), Interferon-stimulated gene - 54kD (ISG-54kD), Guanylate binding protein-1 (GBP-1), MxA and MxB. Expression of these proteins, or their corresponding mRNA, may be used as markers of immunostimulatory activity in cultured cells or tissues or blood of animals to which a CIC has been administered. Expression of such markers can be monitored any of a variety of methods of assessing gene expression, including measurement of mRNAs (e.g., by quantitative PCR), immunoassay (e.g., ELISA), and the like.

[0140] *In vitro* assays can also be carried out using mouse cells, as described, for example, in Example 42, *infra*, and in other mammalian cells.

[0141] Exemplary *in vivo* assays are described in Examples 43, 44, and 46 (mice) and Example 45 (non-human primates).

[0142] Except where otherwise indicated or apparent, the cytokine assays described in the Examples, *infra*, are conducted using human PBMCs using essentially the protocol described in Example 28. Large numbers of test compounds can be assayed simultaneously, e.g., using multi-well plates or other multi-chamber assay materials. If desired, the assays can be carried out by computer-controlled robotic mechanisms well known in the art.

### 3. Nucleic Acid Moieties

[0143] The CICs of the invention comprise one or more nucleic acid moieties. The term "nucleic acid moiety," as used herein, refers to a nucleotide monomer (i.e., a mononucleotide) or polymer (i.e., comprising at least 2 contiguous nucleotides). As used herein, a nucleotide comprises (1) a purine or pyrimidine base linked to a sugar that is in an ester linkage to a phosphate group, or (2) an analog in which the base

and/or sugar and/or phosphate ester are replaced by analogs, *e.g.*, as described *infra*. In a CIC comprising more than one nucleic acid moiety, the nucleic acid moieties may be the same or different.

[0144] The next three sections describe characteristics of nucleic acid moieties such as length, the presence, and the position of sequences or sequence motifs in the moiety, as well as describing (without intending to limit the invention) the properties and structure of nucleic acid moieties and CICs containing the moieties.

#### A. Length

[0145] Usually, a nucleic acid moiety is from 1 to 100 nucleotides in length, although longer moieties are possible in some embodiments. In some embodiments, the length of one or more of the nucleic acid moieties in a CIC is less than 8 nucleotides (*i.e.*, 1, 2, 3, 4, 5, 6 or 7 nucleotides). In various embodiments, a nucleic acid moiety (such as a nucleic acid moiety fewer than 8 nucleotides in length) is at least 2 nucleotides in length, often at least 3, at least 4, at least 5, at least 6, or at least 7 nucleotides in length. In other embodiments, the nucleic acid moiety is at least 10, at least 20, or at least 30 nucleotides in length.

[0146] As shown in the Examples *infra*, CICs containing only heptameric, hexameric, pentameric, tetrameric, and trimeric nucleic acid moieties were active in assays for immunostimulatory activity (*e.g.*, Examples 36 and 37). Thus, it is contemplated that, in some embodiments, a CIC will comprise at least one nucleic acid moiety shorter than 8 nucleotides. In some embodiments, all of the nucleic acid moieties in a CIC will be shorter than 8 nucleotides (*e.g.*, having a length in a range defined by a lower limit of 2, 3, 4, 5, of 6 and an independently selected upper limit of 5, 6, or 7 nucleotides, where the upper limit is higher than the lower limit). For example, in one embodiment, specified nucleic acid moieties in a CIC (including all of the the nucleic acid moieties in the CIC) may be either 6 or 7 nucleotides in length.



In one embodiment, the CIC comprises two spacer moieties and an intervening nucleic acid moiety that is less than 8 bases in length (e.g., 5, 6, or 7 bases in length).

[0147] It is contemplated that in a CIC comprising multiple nucleic acid moieties, the nucleic acid moieties can be the same or different lengths. In one embodiment, the length of one or more, or most (e.g., at least about 2, at least about 4, or at least about 25%, at least about 50%, at least about 75%) or all of the nucleic acid moieties in a CIC is fewer than 8 nucleotides, in some embodiments fewer than 7 nucleotides, in some embodiments fewer than 6 nucleotides, in some embodiments between 2 and 6 nucleotides, in some embodiments between 2 and 7 nucleotides, in some embodiments between 3 and 7 nucleotides, in some embodiments between 4 and 7 nucleotides, in some embodiments between 5 and 7 nucleotides, and in some embodiments between 6 and 7 nucleotides.

[0148] As is discussed in greater detail *infra*, often at least one nucleic acid moiety of a CIC includes the sequence CG, e.g. TCG, or a CG-containing motif described herein. In one embodiment, at least one nucleic acid moiety comprises a CG-containing nucleic acid motif and is less than 8 nucleotides in length (e.g., has a specified length as described *supra* less than 8 nucleotides). In a related embodiment, none of the nucleic acid moieties in a CIC that are longer than 8 nucleotides comprise the sequence "CG" or optionally the sequence "TCG" or "ACG" (i.e., all of the nucleic acid moieties in the CIC that comprise the sequence CG are less than 8 nucleotides in length). In an embodiment, at least one nucleic acid moiety in the CIC does not comprise a CG sequence.

#### B. Sequences and Motifs

[0149] As noted *supra*, a particular nucleic acid moiety can have a variety of lengths. In one embodiment, the nucleic acid moiety has a length shorter than 8 nucleotides. In one embodiment, the nucleic acid moiety has a length of 8

nucleotides or longer. In various embodiments at least one nucleic acid moiety of a CIC of the invention comprises a sequence as disclosed *infra*.

[0150] In the formulas provided below, all sequences are in the 5'→3' direction and the following abbreviations are used: B = 5-bromocytosine; bU = 5-bromouracil; a-A = 2-amino-adenine; g = 6-thio-guanine; t = 4-thio-thymine. H = a modified cytosine comprising an electron-withdrawing group, such as halogen in the 5 position. In various embodiments, a cytosine (C) in a sequence referred to *infra* is replaced with N4-ethylcytosine or N4-methylcytosine or 5-hydroxycytosine. In various embodiments, a guanosine (G) in the formula is replaced with 7-deazaguanosine.

[0151] In CICs tested thus far, the presence of CG correlates with cytokine-inducing activity. Thus, in one embodiment, at least one nucleic acid moiety of a CIC comprises at least one 5'-cytosine, guanine-3' (5'-CG-3') sequence. The cytosine is not methylated at the C-5 position and, preferably is not methylated at any position.

[0152] In one embodiment, one or more nucleic acid moieties comprises 3 to 7 bases. In one embodiment, the nucleic acid moiety comprises 3 to 7 bases and has the sequence 5'-[(X)<sub>0-2</sub>]TCG[(X)<sub>2-4</sub>]-3', or 5'-TCG[(X)<sub>2-4</sub>]-3', or 5'-TCG(A/T)[(X)<sub>1-3</sub>]-3', or 5'-TCG(A/T)CG(A/T)-3', or 5'-TCGACGT-3' or 5'-TCGTCGA-3', wherein each X is an independently selected nucleotide. In some embodiments, the CIC contains at least 3, at least 10, at least 30 or at least 100 nucleic acid moieties having an aforementioned sequence.

[0153] In an embodiment, the nucleic acid moiety comprises the sequence 5'-thymidine, cytosine, guanine-3' (5'-TCG-3'), for example (without limitation), the 3-mer TCG, the 4-mer TCGX (e.g., TCGA), the 5-mers TCGXX (e.g., TCGTC and TCGAT), the 6-mers TCGXXX, XTCGXX and TCGTCG, and the 7-mers TCGXXXX, XTCGXXX, XXTCGXX and TCGTCGX, where X is any base. Often, at least one nucleic acid moiety comprises the sequence 5'-thymidine, cytosine,

guanine, adenosine-3' (5'-TCGA-3'), e.g., comprises a sequence 5'-TCGACGT-3'. In one embodiment, the nucleic acid moiety comprises a heptameric sequence 5'-TCGXCGX, 5'-TCGXTCG, 5'-TCGXXCG, 5'-TCGCGXX where X is any base. In some embodiments the aforementioned sequence is located at the 5-prime position of a CIC, e.g., 5'<sup>F</sup>-TCGXCGX, 5'<sup>F</sup>-TCGXTCG, 5'<sup>F</sup>-TCGXXCG, 5'<sup>F</sup>-TCGCGXX'. CICs comprising these sequences have been discovered to be particularly effective for induction of IFN secretion. For example, compare the results in Figure 10 for C-41 with C-21, C-74 with C-51, and C-143 with C-94, C-142, and C-158.

**[0154]** In some embodiments, a nucleic acid moiety comprises the sequence 5'-ACGTTCG-3'; 5'-TCGTCG-3'; 5'-AACGTTC-3'; 5'-AACGTT-3'; 5'-TCGTT-3'; 5'-CGTTCG-3'; 5'-TCGTCGA-3'; 5'-TCGXXX-3'; 5'-XTCGXX-3'; 5'-XXTCGX-3'; 5'-TCGAGA-3'; 5'-TCGTTT-3'; 5'-TTCGAG-3'; 5'-TTCGT-3'; 5'-TTCGC-3'; 5'-GTCGT-3'; 5'-ATCGT-3'; 5'-ATCGAT-3'; 5'-GTCGTT-3'; 5'-GTCGAC-3'; 5'-ACCGGT-3'; 5'-AABGTT-3'; 5'-AABGUT-3'; 5'-TCGTBG-3' where X is any nucleotide.

**[0155]** In some embodiments, a nucleic acid moiety comprises a sequence that is 5'-purine, purine, C, G, pyrimidine, pyrimidine-3'; 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'; or 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3'; for example (all 5'→3'), GACGCT; GACGTC; GACGTT; GACGCC; GACGCU; GACGUC; GACGUU; GACGUT; GACGTU; AGCGTT; AGCGCT; AGCGTC; AGCGCC; AGCGUU; AGCGCU; AGCGUC; AGCGUT; AGCGTU; AACGTC; AACGCC; AACGTT; AACGCT; AACGUC; AACGUU; AACGCU; AACGUT; AACGTU; GGC GTT; GGC GCT; GGC GTC; GGC GCC; GGC GUU; GGC GCU; GGC GUC; GGC GUT; GGC GTU, AACGTT, AGCGTC, GACGTT, GGC GTT, AACGTC, GACGTC, GGC GTC, AACGCC, AGCGCC, GACGCC, GGC GCC, AGCGCT, GACGCT, GGC GCT, GGC GTT, and AACGCC. In some embodiments, a nucleic acid moiety comprises the sequence: 5'-purine, purine,

cytosine, guanine, pyrimidine, pyrimidine, cytosine, cytosine-3' or 5'-purine, purine, cytosine, guanine, pyrimidine, pyrimidine, cytosine, guanine-3'.

**[0156]** In some embodiments, a nucleic acid moiety comprises a sequence (all 5'→3') AACGTTTCG; AACGTTCC; AACGUTCG; AABGTTTCG; AABGUTCG and/or AABGTTBG.

**[0157]** In various embodiments, a nucleic acid moiety comprises the motif 5'-X<sub>1</sub>X<sub>2</sub>A X<sub>3</sub>C G X<sub>4</sub>T C G-3' (SEQ ID NO:4) wherein X<sub>1</sub> is T, G, C or B, wherein X<sub>2</sub> is T, G, A or U, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U and wherein the sequence is not 5'-TGAACGTTTCG-3' (SEQ ID NO:5) or 5'-GGAACGTTTCG-3' (SEQ ID NO:6). Examples include (all 5'→3'): TGAACGUTCG (SEQ ID NO:7); TGACCGTTTCG (SEQ ID NO:8); TGATCGGTCG (SEQ ID NO:9); TGATCGTTTCG (SEQ ID NO:10); TGAACGGTCG (SEQ ID NO:11); GTAACGTTTCG (SEQ ID NO:12); GTATCGGTCG (SEQ ID NO:13); GTACCGTTTCG (SEQ ID NO:14); GAACCGTTTCG (SEQ ID NO:15); BGACCGTTTCG (SEQ ID NO:16); CGAACGTTTCG (SEQ ID NO:17); CGACCGTTTCG (SEQ ID NO:18); BGAACGTTTCG (SEQ ID NO:19); TTAACGUTCG (SEQ ID NO:20); TUAACGUTCG (SEQ ID NO:21) and TTAACGTTTCG (SEQ ID NO:22).

**[0158]** In various embodiments, a nucleic acid moiety comprises a sequence:

5'-TCGTCGAACGTTTCGTTAACGTTTCG-3' (SEQ ID NO:23);  
5'-TGACTGTGAACGUTCGAGATGA-3' (SEQ ID NO:24);  
5'-TCGTCGAUCGUTC GTTAACGUTCG-3' (SEQ ID NO:25);  
5'-TCGTCGAUCGTTTCGTUAACGUTCG-3' (SEQ ID NO:26);  
5'-TCGTCGUACGUTC GTTAACGUTCG-3' (SEQ ID NO:27);  
5'-TCGTCGAa-ACGUTC GTTAACGUTCG-3' (SEQ ID NO:28);  
5'-TGATCGAACGTTTCGTTAACGTTTCG-3 (SEQ ID NO:29);  
5'-TGACTGTGAACGUTC GGTATGA-3' (SEQ ID NO:30);  
5'-TGACTGTGACCGTTTCGGTATGA-3' (SEQ ID NO:31);  
5'-TGACTGTGATCGGTCGGTATGA-3' (SEQ ID NO:32);

5'-TCGTCGAACGTTTCGTT-3' (SEQ ID NO:33);  
 5'-TCGTCGTGAACGTTTCGAGATGA-3' (SEQ ID NO:34);  
 5'-TCGTCGGTATCGGTCGGTATGA-3' (SEQ ID NO:35);  
 5'-CTTCGAACGTTTCGAGATG-3' (SEQ ID NO:36);  
 5'-CTGTGATCGTTTCGAGATG-3' (SEQ ID NO:37);  
 5'-TGACTGTGAACGGTCGGTATGA-3' (SEQ ID NO:38);  
 5'-TCGTCGGTACCGTTTCGGTATGA-3' (SEQ ID NO:39);  
 5'-TCGTCGGAACCGTTTCGGAATGA-3' (SEQ ID NO:40);  
 5'-TCGTCGAACGTTTCGAGATG-3' (SEQ ID NO:41);  
 5'-TCGTCGTAACGTTTCGAGATG-3' (SEQ ID NO:42);  
 5'-TGACTGTGACCGTTTCGGAATGA-3' (SEQ ID NO:43);  
 5'-TCGTCGAACGTTTCGAACGTTTCG-3' (SEQ ID NO:44);  
 5'-TBGTBGAACGTTTCGAGATG-3' (SEQ ID NO:45);  
 5'-TCGTBGAACGTTTCGAGATG-3' (SEQ ID NO:46);  
 5'-TCGTCGACCGTTTCGGAATGA-3' (SEQ ID NO:47);  
 5'-TBGTBGACCGTTTCGGAATGA-3' (SEQ ID NO:48);  
 5'-TCGTBGACCGTTTCGGAATGA-3' (SEQ ID NO:49);  
 5'-TTCGAACGTTTCGTTAACGTTTCG-3' (SEQ ID NO:50);  
 5'-CTTBGAACGTTTCGAGATG-3' (SEQ ID NO:51);  
 5'-TGATCGTCGAACGTTTCGAGATG-3' (SEQ ID NO:52).

**[0159]** In some embodiments, a nucleic acid moiety comprises the sequence: 5'-X<sub>1</sub>X<sub>2</sub>A X<sub>3</sub>B G X<sub>4</sub>T C G-3' (SEQ ID NO:53), wherein X<sub>1</sub> is T, G, C or B, wherein X<sub>2</sub> is T, G, A or U, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U. In some embodiments, the nucleic acid moiety is not 5'-TGAABGTTTCG-3' (SEQ ID NO:54). Examples include (all 5'→3'): TGAABGUTCG (SEQ ID NO:55); TGACBGTTTCG (SEQ ID NO:56); TGATBGGTTCG (SEQ ID NO:57); GTATBGGTTCG (SEQ ID NO:58); GTACBGTTTCG (SEQ ID NO:59); GAACBGTTTCG (SEQ ID NO:60); GAAABGUTCG (SEQ ID NO:61); BGACBGTTTCG (SEQ ID NO:62);

CGAABGTTCG (SEQ ID NO:63); BGAABGTTCG (SEQ ID NO:64);  
BGAABGUTCG (SEQ ID NO:65); TTAABGUTCG (SEQ ID NO:66);  
TUAABGUTCG (SEQ ID NO:67) and TTAABGTTCG (SEQ ID NO:68).

[0160] In some embodiments, a nucleic acid moiety comprises the sequence:

5'-TGACTGTGAABGUTCGAGATGA-3' (SEQ ID NO:69);  
5'-TCGTCGAABGTTCGTTAABGTTCG-3' (SEQ ID NO:70);  
5'-TGACTGTGAABGUTCGGTATGA-3' (SEQ ID NO:71);  
5'-TGACTGTGAABGUTCGGAATGA-3' (SEQ ID NO:72);  
5'-TCGTCGAAABGUTCGGAATGA-3' (SEQ ID NO:73);  
5'-TCGTBGAABGUTCGGAATGA-3' (SEQ ID NO:74).

[0161] In some embodiments, a nucleic acid moiety comprises the sequence:

5'-X<sub>1</sub>X<sub>2</sub>A.X<sub>3</sub>C.GX<sub>4</sub>TCG-3' (SEQ ID NO:75) wherein X<sub>1</sub> is T, C or B, wherein X<sub>2</sub> is T, G, A or U, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U. In some embodiments, the formula is not 5'-TGAACGTTCG-3' (SEQ ID NO:5)

[0162] In other embodiments, the nucleic acid moiety comprises the sequence:

5'-TGACTGTGAABGTTCGAGATGA-3' (SEQ ID NO:76);  
5'-TGACTGTGAABGTTBGAGATGA-3' (SEQ ID NO:77);  
5'-TGACTGTGAABGTTCAGATGA-3' (SEQ ID NO:78);  
5'-TGACTGTGAACGTUCGAGATGA-3' (SEQ ID NO:79);  
5'-TGACTGTGAACGUTCGAGATGA-3' (SEQ ID NO:80);  
5'-TGACTGTGAABGTTCGTUATGA-3' (SEQ ID NO:81);  
5'-TGACTGTGAABGTTCGGTATGA-3' (SEQ ID NO:82);  
5'-CTGTGAACGTTCGAGATG-3' (SEQ ID NO:83);  
5'-TBGTBGTGAACGTTCGAGATGA-3' (SEQ ID NO:84);  
5'-TCGTBGTGAACGTTCGAGATGA-3' (SEQ ID NO:85);  
5'-TGACTGTGAACGTTCGAGATGA-3' (SEQ ID NO:86);  
5'-TGACTGTGAACgTTCgAGATGA-3' (SEQ ID NO:87);  
5'-TGACTGTGAACGTTCGTUATGA-3' (SEQ ID NO:88);

5'-TGACTGTGAACGTTTCGTTATGA-3' (SEQ ID NO:89);  
 5'-TCGTTCAACGTTTCGTTAACGTTTCG-3' (SEQ ID NO:90);  
 5'-TGATTCAACGTTTCGTTAACGTTTCG-3' (SEQ ID NO:91);  
 5'-CTGTCAACGTTTCGAGATG-3' (SEQ ID NO:92);  
 5'-TCGTCGGAACGTTTCGAGATG-3' (SEQ ID NO:93);  
 5'-TCGTCGGACGTTTCGAGATG-3' (SEQ ID NO:94);  
 5'-TCGTCGTACGTTTCGAGATG-3' (SEQ ID NO:95);  
 5'-TCGTCGTTTCGTTTCGAGATG-3' (SEQ ID NO:96).

**[0163]** In some embodiments, with respect to any of the sequences disclosed *supra*, the nucleic acid moiety further comprises one, two, three or more TCG and/or TBG and/or THG, sequences, preferably 5' to the sequence provided *supra*. The TCG(s) and/or TBG(s) may or may not be directly adjacent to the sequence shown. For example, in some embodiments, a nucleic acid moiety includes any of the following: 5'-TCGTGAACGTTTCG-3' (SEQ ID NO:97); 5'-TCGTCGAACGTTTCG-3' (SEQ ID NO:98); 5'-TBGTGAACGTTTCG-3' (SEQ ID NO:99); 5-TBGTBGAACGTTTCG-3' (SEQ ID NO:100); 5'-TCGTTAACGTTTCG-3' (SEQ ID NO:101). In some embodiments, the additional TCG and/or TBG sequence(s) is immediately 5' and adjacent to the reference sequence. In other embodiments, there is a one or two base separation.

**[0164]** In some embodiments, a nucleic acid moiety has the sequence: 5'-(TCG)<sub>w</sub> N<sub>y</sub> A X<sub>3</sub> C G X<sub>4</sub> T C G-3' (SEQ ID NO:102) wherein w is 1-2, wherein y is 0-2, wherein N is any base, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U.

**[0165]** In some embodiments, the nucleic acid moiety comprises any of the following sequences: TCGAACGTTTCG (SEQ ID NO:103); TCGTCGAACGTTTCG (SEQ ID NO:98); TCGTGAACGTTTCG (SEQ ID NO:97); TCGGTATCGGTTCG (SEQ ID NO:106); TCGGTACCGTTTCG (SEQ ID NO:107); TCGGAACCGTTTCG (SEQ ID NO:108); TCGGAACGTTTCG (SEQ ID NO:109); TCGTCGGAACGTTTCG (SEQ ID NO:110); TCGTAACGTTTCG (SEQ ID NO:111); TCGACCGTTTCG (SEQ

ID NO:112); TCGTCGACCGTTTCG (SEQ ID NO:113); TCGTTAACGTTTCG (SEQ ID NO:101).

**[0166]** In some embodiments, a nucleic acid moiety comprises any of the following sequences: 5'-(TBG)<sub>z</sub> N<sub>y</sub> A X<sub>3</sub> C G X<sub>4</sub> T C G-3' (SEQ ID NO:115) wherein z is 1-2, wherein y is 0-2, wherein B is 5-bromocytosine, wherein N is any base, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U.

**[0167]** In some embodiments, a nucleic acid moiety comprises: TBGTGAACGTTTCG (SEQ ID NO:99); TBGTBGTGAACGTTTCG (SEQ ID NO:117); TBGAACGTTTCG (SEQ ID NO:118); TBGTBGAACGTTTCG (SEQ ID NO:100); TBGACCGTTTCG (SEQ ID NO:119); TBGTBGACCGTTTCG (SEQ ID NO:120).

**[0168]** In some embodiments, a nucleic acid moiety comprises any of the following sequences: 5'-T C G T B G N<sub>y</sub> A X<sub>3</sub> C G X<sub>4</sub> T C G-3' (SEQ ID NO:121) wherein y is 0-2, wherein B is 5-bromocytosine, wherein N is any base, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U. In some embodiments, the nucleic acid moiety comprises any of the following sequences: TCGTBGTGAACGTTTCG (SEQ ID NO:122); TCGTBGAACGTTTCG (SEQ ID NO:123); TCGTBGACCGTTTCG (SEQ ID NO:124).

**[0169]** In some embodiments, a nucleic acid moiety comprises any of the following sequences: 5'-(TCG)<sub>w</sub> N<sub>y</sub> A X<sub>3</sub> B G X<sub>4</sub> T C G-3' (SEQ ID NO:125) wherein w is 1-2, wherein y is 0-2, wherein N is any base, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U. In some embodiments, the nucleic acid moiety comprises any of the following sequences: TCGGAAABGTTTCG (SEQ ID NO:126) or TCGAABGTTTCG (SEQ ID NO:127).

**[0170]** In some embodiments, a nucleic acid moiety comprises any of the following sequences: 5'-(TBG)<sub>z</sub> N<sub>y</sub> A X<sub>3</sub> B G X<sub>4</sub> T C G-3' (SEQ ID NO:128) wherein z is 1-2, wherein y is 0-2, wherein B is 5-bromocytosine, wherein N is any base, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U. In some embodiments, the



nucleic acid moiety comprises any of the following sequences: TBGAABGUTCG (SEQ ID NO:129) or TBGAABGTTCG (SEQ ID NO:130).

**[0171]** In some embodiments, a nucleic acid moiety comprises any of the following sequences: 5'-T C G T B G N<sub>y</sub> A X<sub>3</sub> B G X<sub>4</sub> T C G-3' (SEQ ID NO:131) wherein y is 0-2, wherein B is 5-bromocytosine, wherein N is any base, wherein X<sub>3</sub> is T, A or C, wherein X<sub>4</sub> is T, G or U. In some embodiments, the nucleic acid moiety comprises any of the following sequences: TCGTBGAABGUTCG (SEQ ID NO:132) or TCGTBGAABGTTCG (SEQ ID NO:133).

**[0172]** In some embodiments, a nucleic acid moiety comprises the sequence: AACGTTCC, AACGTTCCG, GACGTTCC, GACGTTCCG.

**[0173]** In some embodiments, a nucleic acid moiety comprises the sequence: GCGGTTCCG; GCGGCTCCG; GCGGTTCCG; GCGGCCCG; GACGTTCC; GACGCTCC; GACGTCCC; GACGCCCC; AGCGTTCC; AGCGCTCC; AGCGTCCC; AGCGCCCC; AACGTTCC; AACGCTCC; AACGTCCC; AACGCCCC; GCGGTTCC; GCGGCTCC; GCGGTCCC; GCGGCCCG; GACGTTCCG; GACGCTCCG; GACGTCCG; GACGCCCCG; AGCGTTCCG; AGCGCTCCG; AGCGTCCG; AGCGCCCCG; AACGTTCCG; AACGCTCCG; AACGTCCG; AACGCCCCG; GACGCTCC; GACGCCC; AGCGTTCC; AGCGCTCC; AGCGTCCC; AGCGCCCC; AACGTCCC; AACGCCCC; GCGGTTCC; GCGGCTCC; GCGGTCCC; GCGGCCCG; GACGCTCCG; GACGTCCG; GACGCCCCG; AGCGTTCCG; AGCGTCCG; AGCGCCCCG; AACGTCCG; AACGCCCCG.

**[0174]** In some embodiments, a nucleic acid moiety comprises the sequence: (5'→3') TCGTCGA; TCGTCG; TCGTTT; TTCGTT; TTTTCG; ATCGAT; GTCGAC; GTCGTT; TCGCGA; TCGTTTT; TCGTC; TCGTT; TCGT; TCG; ACGTTT; CCGTTT; GCGTTT; AACGTT; TCGAAAA; TCGCCCC; TCGGGGG.

**[0175]** In some embodiments, a nucleic acid moiety comprises an RNA of the sequence AACGUUCC, AACGUUCG, GACGUUCC, and GACGUUCG.

[0176] In some embodiments, a nucleic acid moiety has a sequence comprising a sequence or sequence motif described in copending coassigned U.S. patent applications 09/802,685 (published as U.S. Application Publication No. 20020028784A1 on March 7, 2002 and as WO 01/68077 on September 20, 2001); 09/802,359 (published as WO 01/68144 on September 20, 2001), and copending U.S. Application Serial No. 10/033,243, or in PCT publications WO 97/28259, WO 98/16247; WO 98/55495; WO 99/11275; WO 99/62923; and WO 01/35991. The nucleic acid moiety can also have a sequence comprising any or several of the sequences previously reported to be correlated with immunostimulatory activity when administered as a polynucleotide greater (often substantially greater) than 8 nucleotides in length, e.g., Kandimalla et al., 2001, *Bioorg. Med. Chem.* 9:807-13; Krieg et al. (1989) *J. Immunol.* 143:2448-2451; Tokunaga et al. (1992) *Microbiol. Immunol.* 36:55-66; Kataoka et al. (1992) *Jpn. J. Cancer Res.* 83:244-247; Yamamoto et al. (1992) *J. Immunol.* 148:4072-4076; Mojcik et al. (1993) *Clin. Immuno. and Immunopathol.* 67:130-136; Branda et al. (1993) *Biochem. Pharmacol.* 45:2037-2043; Pisetsky et al. (1994) *Life Sci.* 54(2):101-107; Yamamoto et al. (1994a) *Antisense Research and Development.* 4:119-122; Yamamoto et al. (1994b) *Jpn. J. Cancer Res.* 85:775-779; Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523; Kimura et al. (1994) *J. Biochem. (Tokyo)* 116:991-994; Krieg et al. (1995) *Nature* 374:546-549; Pisetsky et al. (1995) *Ann. N.Y. Acad. Sci.* 772:152-163; Pisetsky (1996a) *J. Immunol.* 156:421-423; Pisetsky (1996b) *Immunity* 5:303-310; Zhao et al. (1996) *Biochem. Pharmacol.* 51:173-182; Yi et al. (1996) *J. Immunol.* 156:558-564; Krieg (1996) *Trends Microbiol.* 4(2):73-76; Krieg et al. (1996) *Antisense Nucleic Acid Drug Dev.* 6:133-139; Klinman et al. (1996) *Proc. Natl. Acad. Sci. USA.* 93:2879-2883; Raz et al. (1996); Sato et al. (1996) *Science* 273:352-354; Stacey et al. (1996) *J. Immunol.* 157:2116-2122; Ballas et al. (1996) *J. Immunol.* 157:1840-1845; Branda et al. (1996) *J. Lab. Clin. Med.* 128:329-338; Sonehara et al. (1996) *J. Interferon and Cytokine Res.* 16:799-803; Klinman et al. (1997) *J. Immunol.*

158:3635-3639; Sparwasser et al. (1997) *Eur. J. Immunol.* 27:1671-1679; Roman et al. (1997) *Nat Med.* 3:849-54; Carson et al. (1997) *J. Exp. Med.* 186:1621-1622; Chace et al. (1997) *Clin. Immunol. and Immunopathol.* 84:185-193; Chu et al. (1997) *J. Exp. Med.* 186:1623-1631; Lipford et al. (1997a) *Eur. J. Immunol.* 27:2340-2344; Lipford et al. (1997b) *Eur. J. Immunol.* 27:3420-3426; Weiner et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:10833-10837; Macfarlane et al. (1997) *Immunology* 91:586-593; Schwartz et al. (1997) *J. Clin. Invest.* 100:68-73; Stein et al. (1997) *Antisense Technology*, Ch. 11 pp. 241-264, C. Lichtenstein and W. Nellen, Eds., IRL Press; Wooldridge et al. (1997) *Blood* 89:2994-2998; Leclerc et al. (1997) *Cell. Immunol.* 179:97-106; Kline et al. (1997) *J. Invest. Med.* 45(3):282A; Yi et al. (1998a) *J. Immunol.* 160:1240-1245; Yi et al. (1998b) *J. Immunol.* 160:4755-4761; Yi et al. (1998c) *J. Immunol.* 160:5898-5906; Yi et al. (1998d) *J. Immunol.* 161:4493-4497; Krieg (1998) *Applied Antisense Oligonucleotide Technology* Ch. 24, pp. 431-448, C.A. Stein and A.M. Krieg, Eds., Wiley-Liss, Inc.; Krieg et al. (1998a) *Trends Microbiol.* 6:23-27; Krieg et al. (1998b) *J. Immunol.* 161:2428-2434; Krieg et al. (1998c) *Proc. Natl. Acad. Sci. USA* 95:12631-12636; Spiegelberg et al. (1998) *Allergy* 53(45S):93-97; Horner et al. (1998) *Cell Immunol.* 190:77-82; Jakob et al. (1998) *J. Immunol.* 161:3042-3049; Redford et al. (1998) *J. Immunol.* 161:3930-3935; Weeratna et al. (1998) *Antisense & Nucleic Acid Drug Development* 8:351-356; McCluskie et al. (1998) *J. Immunol.* 161(9):4463-4466; Gramzinski et al. (1998) *Mol. Med.* 4:109-118; Liu et al. (1998) *Blood* 92:3730-3736; Moldoveanu et al. (1998) *Vaccine* 16: 1216-1224; Brazolot Milan et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:15553-15558; Briode et al. (1998) *J. Immunol.* 161:7054-7062; Briode et al. (1999) *Int. Arch. Allergy Immunol.* 118:453-456; Kovarik et al. (1999) *J. Immunol.* 162:1611-1617; Spiegelberg et al. (1999) *Pediatr. Pulmonol. Suppl.* 18:118-121; Martin-Orozco et al. (1999) *Int. Immunol.* 11:1111-1118; EP 468,520; WO 96/02555; WO 97/28259; WO 98/16247; WO 98/18810; WO 98/37919; WO 98/40100; WO 98/52581; WO 98/55495; WO 98/55609 and WO 99/11275. See also

Elkins et al. (1999) *J. Immunol.* 162:2291-2298, WO 98/52962, WO 99/33488, WO 99/33868, WO 99/51259 and WO 99/62923. See also Zimmermann et al. (1998) *J. Immunol.* 160:3627-3630; Krieg (1999) *Trends Microbiol.* 7:64-65 and U.S. Patent Nos. 5,663,153, 5,723,335 and 5,849,719. See also Liang et al. (1996) *J. Clin. Invest.* 98:1119-1129; Bohle et al. (1999) *Eur. J. Immunol.* 29:2344-2353 and WO 99/56755. See also WO 99/61056; WO 00/06588; WO 00/16804; WO 00/21556; WO 00/54803; WO 00/61151; WO 00/67023; WO 00/67787 and U.S. Patent No. 6,090,791. In one embodiment, at least one nucleic acid moiety of a CIC comprises a TG sequence or a pyrimidine-rich (e.g., T-rich or C-rich) sequence, as described in PCT publication WO 01/22972.

[0177] In some embodiments, the nucleic acid moiety is other than one or more of the hexamers 5'-GACGTT-3', 5'-GAGCTT-3', 5'-TCCGGA-3', 5'-AACGTT-3', 5'-GACGTT-3', 5'-TACGTT-3', 5'-CACGTT-3', 5'-AGCGTT-3', 5'-ATCGTT-3', 5'-ACCGTT-3', 5'-AACGGT-3', 5'-AACGAT-3', 5'-AACGCT-3', 5'-AACGTG-3', 5'-AACGTA-3', and 5'-AACGTC-3'.

[0178] In some embodiments, the CIC contains at least 3, at least 10, at least 30 or at least 100 nucleic acid moieties having a sequence described above.

#### C. Nucleic Acid Moiety Sequences: Heterogeneity and Position

[0179] It is contemplated that in a CIC comprising multiple nucleic acid moieties, the nucleic acid moieties can be the same or different.

[0180] In one embodiment, all of the nucleic acid moieties in a CIC have the same sequence. In one embodiment, a CIC comprises nucleic acid moieties with at least 2, at least 3, at least 4, at least 5, or at least 6 or more different sequences. In one embodiment, a CIC has fewer than 10 different nucleic acid moieties. In one embodiment each of the nucleic acid moieties in a CIC has a different sequence.

[0181] In some embodiments, a single nucleic acid moiety contains more than one iteration of a sequence motif listed above in §3(B), or two or more different

sequence motifs. The motifs within a single nucleic acid moiety can be adjacent, overlapping, or separated by additional nucleotide bases within the nucleic acid moiety. In an embodiment, a nucleic acid moiety includes one or more palindromic regions. In the context of single-stranded oligonucleotides, the term "palindromic" refers to a sequence that would be palindromic if the oligonucleotide were complexed with a complementary sequence to form a double-stranded molecule. In another embodiment, one nucleic acid moiety has a sequence that is palindromic or partially palindromic in relation to a second nucleic acid moiety in the CIC. In an embodiment of the invention, the sequence of one or more of the nucleic acid moieties of a CIC is not palindromic. In an embodiment of the invention, the sequence of one or more of the nucleic acid moieties of a CIC does not include a palindromic sequence greater than four bases, optionally greater than 6 bases.

**[0182]** As described *supra*, in various embodiments, one or more (*e.g.*, all) of the nucleic acid moieties in a CIC comprises a 5'-CG-3' sequence, alternatively a 5'-TCG-3' sequence. In one embodiment, the nucleic acid moiety is 5, 6 or 7 bases in length. In an embodiment, the nucleic acid moiety has the formula 5'-TCG[(X)<sub>2-4</sub>]-3' or 5'-TCG(A/T)[(X)<sub>1-3</sub>] or 5'-TCG(A/T)CG(A/T)-3' or 5'-TCGACGT-3' (where each X is an independently selected nucleotide). In one embodiment, the aforementioned nucleic acid moiety is a 5-prime moiety.

**[0183]** In one embodiment, a nucleic acid moiety comprises a sequence 5'-TCGTCGA-3'. In one embodiment, a nucleic acid moiety comprises a sequence selected from (all 5'→3'): TCGXXXX, TCGAXXX, XTCGXXX, XTCGAXX, TCGACGT, TCGAACG, TCGAGAT, TCGACTC, TCGAGCG, TCGATTT, TCGCTTT, TCGGTTT, TCGTTTT, TCGTCGT, ATCGATT, TTCGTTT, TTCGATT, ACGTTCG, AACGTTC, TGACGTT, TGTCGTT, TCGXXX, TCGAXX, GTCGTT, GACGTT, ATCGAT, TCGTCG; TCGTTT; TCGAGA; TTCGAG; TTCGTT; AACGTT; AACGTTCG; AACGUTCG, ABGUTCG,

TCGXX, TCGAX, TCGAT, TCGTT, TCGTC; TCGA, TCGT, and TCGX (where X is A, T, G or C; U is 2'-deoxyuridine and B is 5-bromo-2'-deoxycytidine).

[0184] In one embodiment, the nucleic acid moiety is a 7-mer having the sequence TCGXXXX, TCGAXXX, XTCGXXX, XTCGAXX, TCGTCGA, TCGACGT, TCGAACG, TCGAGAT, TCGACTC, TCGAGCG, TCGATTT, TCGCTTT, TCGGTTT, TCGTTTT, TCGTCGT, ATCGATT, TTCGTTT, TTCGATT, ACGTTCG, AACGTTC, TGACGTT, or TGTCGTT; or is a 6-mer having the sequence TCGXXX, TCGAXX, TCGTCG, AACGTT, ATCGAT, GTCGTT, or GACGTT; or is a 5-mer having the sequence TCGXX, TCGAX, TCGAT, TCGTT, or TCGTC; or is a 4-mer having the sequence TCGA, TCGT, or TCGX, or is a 3-mer having the sequence TCG, where X is A, T, G or C.

[0185] In one embodiment, at least about 25%, preferably at least about 50%, or at least about 75%, and sometimes all of the nucleic acid moieties in the CIC comprise at least one of the aforementioned sequences. In one embodiment, at least one nucleic acid moiety does not comprise a CG motif. In other embodiments, at least about 25%, sometimes at least about 50%, and sometimes at least about 75% of the nucleic acid moieties in the CIC are nucleic acid moieties that do not have a CG motif or, alternatively, a TCG motif.

[0186] The position of a sequence or sequence motif in a CIC can influence the immunomodulatory activity of the CIC, as is illustrated in the Examples, *infra*. In referring to the position of a sequence motif in a nucleic acid moiety of a CIC, the following terminology can be used: (1) In a CIC containing multiple nucleic acid moieties, a moiety with a free-5' end is referred to as "a 5-prime moiety." It will be appreciated that a single CIC may have multiple 5-prime moieties. (2) Within any particular nucleic acid moiety, a sequence or motif is in "the 5-prime position" of the moiety when there are no nucleotide bases 5' to the reference sequence in that moiety. Thus, in the moiety with the sequence 5'-TCGACGT-3', the sequences T, TC, TCG and TCGA, are in "the 5-prime position," while the sequence GAC is not.

By way of illustration, a CIC containing the sequence TCG in the 5-prime position of a nucleic acid moiety can render the CIC more active than an otherwise similar CIC with a differently positioned TCG motif. A CIC with a TCG sequence in a 5-prime moiety, e.g., at the 5-prime position of the 5-prime moiety can render a CIC particularly active. See e.g. Example 38. A nucleic acid moiety with a free 5' end can be designated using the symbol "5'<sup>F</sup>" to the left of the formula for the base sequence of the nucleic acid moiety (e.g., 5'<sup>F</sup>-TACG-3'). As used herein, the term "free 5' end" in the context of a nucleic acid moiety has its usual meaning and means that the 5' terminus of the nucleic acid moiety is not conjugated to a blocking group or a non-nucleotide spacer moiety. The free 5'-nucleoside contains an unmodified 5'-hydroxy group or a 5'-phosphate, 5'-diphosphate, or 5'-triphosphate group, or other common modified phosphate groups (such as thiophosphate, dithiophosphate, and the like) that is not further linked to a blocking or functional group.

**[0187]** Immunostimulatory activity can also be influenced by the position of a CG motif in a nucleic acid moiety (e.g., in a 5'-moiety). For example, in one embodiment the CIC contains at least one nucleic acid moiety with the sequence 5'-X-CG-Y-3' where X is zero, one, or two nucleotides and Y is 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more than 15 nucleotides in length. In an embodiment, the 5'-X-CG-Y-3' sequence is in a 5'-moiety of the CIC, e.g., the 5-prime position of the CIC. In an embodiment, the CIC contains 2, 3 or more nucleic acid moieties with a sequence having the formula 5'-X-CG-Y-3' sequence. For example, in an embodiment, all of the nucleic acid moieties of the CIC have sequences of the formula 5'-X-CG-Y-3' sequence.

**[0188]** Similarly, a CIC including the sequence TCGA (e.g., a sequence including TCGACGT) in a nucleic acid moiety has immunomodulatory activity, and is effective in IFN- $\alpha$  induction. A TCGA (e.g., a sequence including TCGACGT) in a 5-prime moiety, e.g., at the 5-prime position of the 5-prime moiety, renders the CIC particularly active. See examples 38 and 49. Thus, in one embodiment, a CIC

comprises a core structure with the formula (5'-N<sub>1</sub>-3')-S<sub>1</sub>-N<sub>2</sub> (Ia) where N<sub>1</sub> has the sequence 5'-TCGAX-3' and X is 0 to 20 nucleotide bases, often 0 to 3 bases. In one embodiment, X is CGT. The sequence TCGTCGA is also particularly effective in IFN- $\alpha$  induction.

[0189] In addition, the presence of free (unconjugated) nucleic acid 5'-ends can affect immunostimulatory activity. See, e.g., Example 39. In various embodiments, a CIC of the invention comprises at least 1, at least 2, at least 3, at least 4, or at least 5 free 5'-ends. In some embodiments, the number of free 5'-ends is from 1 to 10, from 2 to 6, from 3 to 5, or from 4-5. In one embodiment, the number of free 5' ends is at least about 50 or at least about 100.

#### D. "Isolated Immunomodulatory Activity"

[0190] One property of a nucleic acid moiety is the "isolated immunomodulatory activity" associated with the nucleotide sequence of the nucleic acid moiety. As noted *supra*, the present inventors have discovered that, surprisingly, CICs exhibit immunomodulatory activity even when none of the nucleic acid moieties of the CIC has a sequence that, if presented as a polynucleotide alone, exhibits comparable immunomodulatory activity.

[0191] In some embodiments, a nucleic acid moiety of a CIC does not have "isolated immunomodulatory activity," or has "inferior isolated immunomodulatory activity," (i.e., when compared to the CIC), as described below.

[0192] The "isolated immunomodulatory activity" of a nucleic acid moiety is determined by measuring the immunomodulatory activity of an isolated polynucleotide having the primary sequence of the nucleic acid moiety, and having the same nucleic acid backbone (e.g., phosphorothioate, phosphodiester, chimeric). For example, a CIC having the structure "5'-TCGTCG-3'-HEG-5'-ACGTTCG-3'-HEG-5'-AGATGAT-3'" contains three nucleic acid moieties. To determine the independent immunomodulatory activity of, for example, the first nucleic acid moiety



in the CIC, a test polynucleotide having the same sequence (i.e., 5'-TCGTCG-3') and same backbone structure (e.g., phosphorothioate) is synthesized using routine methods, and its immunomodulatory activity (if any) is measured.

Immunomodulatory activity can be determined using standard assays which indicate various aspects of the immune response, such as those described in §2, *supra*.

Preferably the human PBMC assay described in §2, *supra*, is used. As discussed *supra*, to account for donor variation, typically the assay is carried out using cells obtained from multiple donors. A polynucleotide does not have immunomodulatory activity (and the corresponding nucleic acid moiety does not have "isolated immunomodulatory activity") when the amount of IFN- $\gamma$  secreted by PBMCs contacted with the polynucleotide is not significantly greater (e.g., less than about 2-fold greater) in the majority of donors than in the absence of the test compound or, (in some embodiments) in the presence of an inactive control compound (e.g., 5'-TGACTGTGAACCTTAGAGATGA-3' (SEQ ID NO:3)).

[0193] To compare the immunomodulatory activity of a CIC and an isolated polynucleotide, immunomodulatory activity is measured, preferably using the human PBMC assay described in §2, *supra*. Usually, the activity of two compounds is compared by assaying them in parallel under the same conditions (e.g., using the same cells), usually at a concentration of about 20  $\mu\text{g/ml}$ . As noted *supra*, typically, concentration is determined by measuring absorbance at 260 nm and using the conversion  $0.5 \text{ OD}_{260}/\text{ml} = 20 \mu\text{g/ml}$ . This normalizes the amount of total nucleic acid in the test sample. Alternatively, concentration or weight can be measured by other methods known in the art. If desired, the amount of nucleic acid moiety can be determined by measuring absorbance at 260, and the weight of the CIC calculated using the molecular formula of the CIC. This method is sometimes used when the ratio of weight contributed by the spacer moiety(s) to weight contributed by the nucleic acid moieties in a CIC is high (i.e., greater than 1).

[0194] Alternatively, a concentration of 3  $\mu$ M may be used, particularly when the calculated molecular weights of two samples being compared differ by more than 20%.

[0195] A nucleic acid moiety of a CIC is characterized as having "inferior immunomodulatory activity," when the test polynucleotide has less activity than the CIC to which it is compared. Preferably the isolated immunomodulatory activity of the test polynucleotide is no more than about 50% of the activity of the CIC, more preferably no more than about 20%, most preferably no more than about 10% of the activity of the CIC, or in some embodiments, even less.

[0196] For CICs with multiple (*e.g.*, multiple different) nucleic acid moieties, it is also possible to determine the immunomodulatory activity (if any) of a mixture of test polynucleotides corresponding to the multiple nucleic acid moieties. The assay can be carried out using a total amount of test polynucleotide (*i.e.*, in the mixture) which equals the amount of CIC used. Alternatively, an amount of each test polynucleotide, or each different test polynucleotide, in the mixture can be equal to the amount of the CIC in the assay. As noted in §2, to account for donor variation, preferably assays and analysis use PMBCs from multiple donors.

[0197] In one embodiment, one or more (*e.g.*, at least about 2, at least about 4, or at least about 25%, at least about 50%, or all, measured individually or, alternatively, in combination) of the nucleic acid moieties in a CIC do not have isolated immunomodulatory activity. In one embodiment, one or more (*e.g.*, at least about 2, at least about 4, or at least about 25%, at least about 50%, or all, measured individually or, alternatively, in combination) has inferior isolated immunomodulatory activity compared to the CIC.

[0198] In a related embodiment, a CIC comprises one or more nucleic acid moieties with isolated immunomodulatory activity. For example, in some embodiments, all or almost all (*e.g.*, at least 90%, preferably at least 95%) of the nucleic acid moieties has isolated immunomodulatory activity. For example, a CIC

comprising a multivalent spacer(s) can comprise more than 4, often more than 10, frequently at least about 20, at least about 50, at least about 100, at least about 400 or at least about 1000 nucleic acid moieties (e.g., at least about 2500) with isolated immunostimulatory activity (e.g., having the sequence 5'-TGA CTG TGA ACG TTC GAG ATG A-3' (SEQ ID NO:2)).

[0199] Thus, in a particular CIC, the number of nucleic acid moieties that have isolated immunomodulatory activity can be zero (0), one (1), 2 or more, 3 or more, fewer than 3, 4 or more, fewer than 4, 5 or more, fewer than 5, at least 10, at least about 20, at least about 50, at least about 100, at least about 400 or at least about 1000, all, or less than all, of the nucleic acid moieties of the CIC.

#### E. Structure of the Nucleic Acid Moiety

[0200] A nucleic acid moiety of a CIC may contain structural modifications relative to naturally occurring nucleic acids. Modifications include any known in the art for polynucleotides, but are not limited to, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group. Various such modifications are described below.

[0201] The nucleic acid moiety may be DNA, RNA or mixed DNA/RNA, single stranded, double stranded or partially double stranded, and may contain other modified polynucleotides. Double stranded nucleic acid moieties and CICs are contemplated, and the recitation of the term "base" or "nucleotide" is intended to encompass basepair or basepaired nucleotide, unless otherwise indicated. A nucleic acid moiety may contain naturally-occurring or modified, non-naturally occurring bases, and may contain modified sugar, phosphate, and/or termini. For example, phosphate modifications include, but are not limited to, methyl phosphonate, phosphorothioate, phosphoramidate (bridging or non-bridging), phosphotriester and phosphorodithioate and may be used in any combination. Other non-phosphate

linkages may also be used. Preferably, CICs and nucleic acid moieties of the present invention comprise phosphorothioate backbones. Sugar modifications known in the field, such as 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras and others described herein, may also be made and combined with any phosphate modification. Examples of base modifications (discussed further below) include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine (e.g., 5-bromocytosine, 5-chlorocytosine, 5-fluorocytosine, 5-iodocytosine) and C-5 and/or C-6 of a uracil (e.g., 5-bromouracil, 5-chlorouracil, 5-fluorouracil, 5-iodouracil). See, for example, PCT Application No. WO 99/62923.

[0202] The nucleic acid moiety can also contain phosphate-modified nucleotides. Synthesis of nucleic acids containing modified phosphate linkages or non-phosphate linkages is also known in the art. For a review, see Matteucci (1997) "Oligonucleotide Analogs: an Overview" in *Oligonucleotides as Therapeutic Agents*, (D.J. Chadwick and G. Cardew, ed.) John Wiley and Sons, New York, NY. The phosphorous derivative (or modified phosphate group) which can be attached to the sugar or sugar analog moiety in the nucleic acids of the present invention can be a monophosphate, diphosphate, triphosphate, alkylphosphonate, phosphorothioate, phosphorodithioate, phosphoramidate or the like. The preparation of the above-noted phosphate analogs, and their incorporation into nucleotides, modified nucleotides and oligonucleotides, per se, is also known and need not be described here in detail. Peyrottes et al. (1996) *Nucleic Acids Res.* 24:1841-1848; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24:2318-2323; and Schultz et al. (1996) *Nucleic Acids Res.* 24:2966-2973. For example, synthesis of phosphorothioate oligonucleotides is similar to that described above for naturally occurring oligonucleotides except that the oxidation step is replaced by a sulfurization step (Zon (1993) "Oligonucleoside Phosphorothioates" in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties* (Agrawal, ed.) Humana Press, pp. 165-190). Similarly the synthesis of other phosphate analogs,

such as phosphotriester (Miller et al. (1971) *JACS* 93:6657-6665), non-bridging phosphoramidates (Jager et al. (1988) *Biochem.* 27:7247-7246), N3' to P5' phosphoramidates (Nelson et al. (1997) *JOC* 62:7278-7287) and phosphorodithioates (U.S. Patent No. 5,453,496) has also been described. Other non-phosphorous based modified nucleic acids can also be used (Stirchak et al. (1989) *Nucleic Acids Res.* 17:6129-6141). Nucleic acids with phosphorothioate backbones appear to be more resistant to degradation after injection into the host. Braun et al. (1988) *J. Immunol.* 141:2084-2089; and Latimer et al. (1995) *Mol. Immunol.* 32:1057-1064.

[0203] Nucleic acid moieties used in the invention can comprise ribonucleotides (containing ribose as the only or principal sugar component), and/or deoxyribonucleotides (containing deoxyribose as the principal sugar component). Modified sugars or sugar analogs can be incorporated in the nucleic acid moiety. Thus, in addition to ribose and deoxyribose, the sugar moiety can be pentose, deoxypentose, hexose, deoxyhexose, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar can be in pyranosyl or in a furanosyl form. The sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-O-alkylribose, and the sugar can be attached to the respective heterocyclic bases either in  $\alpha$  or  $\beta$  anomeric configuration. Sugar modifications include, but are not limited to, 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs and 2'-alkoxy- or amino-RNA/DNA chimeras. For example, a sugar modification in the CIC includes, but is not limited to, 2'-amino-2'-deoxyadenosine. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) per se is known, and need not be described here, except to the extent such preparation can pertain to any specific example. Sugar modifications may also be made and combined with any phosphate modification in the preparation of a CIC.

[0204] The heterocyclic bases, or nucleic acid bases, which are incorporated in the nucleic acid moiety can be the naturally-occurring principal purine and

pyrimidine bases, (namely uracil, thymine, cytosine, adenine and guanine, as mentioned above), as well as naturally-occurring and synthetic modifications of said principal bases.

**[0205]** Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) are available in the art, and that as long as other criteria of the present invention are satisfied, the nucleic acid moiety can include one or several heterocyclic bases other than the principal five base components of naturally-occurring nucleic acids. Preferably, however, the heterocyclic base is, without limitation, uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2,3-d] pyrimidin-5-yl, or 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the nucleic acid moiety via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

**[0206]** The nucleic acid moiety may comprise at least one modified base. As used herein, the term "modified base" is synonymous with "base analog", for example, "modified cytosine" is synonymous with "cytosine analog." Similarly, "modified" nucleosides or nucleotides are herein defined as being synonymous with nucleoside or nucleotide "analogs." Examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a cytosine of the nucleic acid moiety. Preferably, the electron-withdrawing moiety is a halogen. Such modified cytosines can include, but are not limited to, azacytosine, 5-bromocytosine, 5-chlorocytosine, chlorinated cytosine, cyclocytosine, cytosine arabinoside, 5-fluorocytosine, fluoropyrimidine, 5,6-dihydrocytosine, 5-iodocytosine, 5-nitrocytosine, and any other pyrimidine analog or modified pyrimidine. Other examples of base modifications include, but are not limited to, addition of an electron-withdrawing moiety to C-5 and/or C-6 of a uracil of the nucleic acid moiety.

Preferably, the electron-withdrawing moiety is a halogen. Such modified uracils can include, but are not limited to, 5-bromouracil, 5-chlorouracil, 5-fluorouracil, 5-iodouracil. Also see, Kandimalla et al., 2001, *Bioorg. Med. Chem.* 9:807-13.

[0207] Other examples of base modifications include the addition of one or more thiol groups to the base including, but not limited to, 6-thio-guanine, 4-thio-thymine and 4-thio-uracil.

[0208] The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Patents 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also been described (including, but not limited to, *e.g.*, U.S. Patents 4,849,513, 5,015,733, 5,118,800, 5,118,802) and can be used similarly.

#### 4. Non-Nucleic Acid Spacer Moieties

[0209] The CIC compounds of the invention comprise one or more non-nucleic acid spacer moieties covalently bound to the nucleic acid moieties. For convenience, non-nucleic acid spacer moieties are sometimes referred to herein simply as "spacers" or "spacer moieties."

[0210] Spacers are generally of molecular weight about 50 to about 500,000 (*e.g.* about 50 to about 50,000), sometimes from about 75 to about 5000, sometimes from about 75 to about 500, which are covalently bound, in various embodiments, to one, two, three, or more than three nucleic acid moieties. A variety of agents are suitable for connecting nucleic acid moieties. For example, a variety of compounds referred to in the scientific literature as "non-nucleic acid linkers," "non-nucleotidic linkers,"

or "valency platform molecules" may be used as spacers in a CIC. A spacer moiety is said to comprise a particular spacer component (e.g., hexaethylene glycol) when the spacer includes the component (or a substituted derivative) as a subunit or portion of the spacer. For example, the spacer shown in Example 49 can be described as comprising a polysaccharide component, a hexaethylene glycol component, and a derivatized thioether linker component. As described *infra*, in certain embodiments, a spacer comprises multiple covalently connected subunits and may have a homopolymeric or heteropolymeric structure. Often the subunits are connected by a linker, phosphodiester linkage, and/or phosphorothioate ester linkage. See the Examples, *infra*. Nonnucleotide spacer moieties of a CIC comprising or derived from such multiple units can be referred to as "compound spacers." In one embodiment, for illustration and not limitation, the CIC comprises a compound spacer comprising any two or more (e.g., 3 or more, 4 or more, or 5 or more) of the following compounds in phosphodiester linkage and/or phosphorothioate ester linkage: oligoethylene glycol unit (e.g., triethylene glycol spacer; hexaethylene glycol spacer); alkyl unit (e.g., propyl spacer; butyl spacer; hexyl spacer); 2-(hydroxymethyl)ethyl spacer; glycerol spacer; trebler spacer; symmetrical doubler spacer.

[0211] It will be appreciated that mononucleotides and polynucleotides are not included in the definition of non-nucleic acid spacers, without which exclusion there would be no difference between nucleic acid moiety and an adjacent non-nucleic acid spacer moiety.

[0212] A variety of spacers are described herein, for illustration and not limitation. It will be appreciated by the reader that, for convenience, a spacer moiety (or component of a spacer moiety) is sometimes referred to by the chemical name of the compound (e.g., hexaethylene glycol) from which the spacer moiety or component is derived, with the understanding that the CIC actually comprises the conjugate of the compound(s) to nucleic acid moieties. As will be understood by the ordinarily skilled practitioner (and as described in greater detail hereinbelow), the



non-nucleic acid spacer can be (and usually is) formed from a spacer moiety precursor(s) that include reactive groups to permit coupling of one more nucleic acid (e.g., oligonucleotides) to the spacer moiety precursor to form the CIC and protecting groups may be included. The reactive groups on the spacer precursor may be the same or different.

[0213] Exemplary non-nucleic acid spacers comprise oligo-ethylene glycol (e.g., triethylene glycol, tetraethylene glycol, hexaethylene glycol spacers, and other polymers comprising up to about 10, about 20, about 40, about 50, about 100 or about 200 ethylene glycol units), alkyl spacers (e.g., propyl, butyl, hexyl, and other C2 – C12 alkyl spacers, e.g., usually C2 – C10 alkyl, most often C2 – C6 alkyl), symmetric or asymmetric spacers derived from glycerol, pentaerythritol, 1,3,5-trihydroxycyclohexane or 1,3-diamino-2-propanol (e.g., symmetrical doubler and trebler spacer moieties described herein). Optionally these spacer components are substituted. For example, as will be understood by one of ordinary skill in the art, glycerol and 1,3-diamino-2-propanol may be substituted at the 1, 2, and/or 3 position (e.g., replacement of one or more hydrogens attached to carbon with one of the groups listed below). Similarly, pentaerythritol may be substituted at any, or all, of the methylene positions with any of the groups described below. Substituents include alcohol, alkoxy (such as methoxy, ethoxy, and propoxy), straight or branched chain alkyl (such as C1-C12 alkyl, preferably C1-C10 alkyl), amine, aminoalkyl (such as amino C1-C12 alkyl, preferably amino C1-C10 alkyl), phosphoramidite, phosphate, phosphoramidate, phosphorodithioate, thiophosphate, hydrazide, hydrazine, halogen, (such as F, Cl, Br, or I), amide, alkylamide (such as amide C1-C12 alkyl, preferably C1-C10 alkyl), carboxylic acid, carboxylic ester, carboxylic anhydride, carboxylic acid halide, ether, sulfonyl halide, imidate ester, isocyanate, isothiocyanate, haloformate, carbodiimide adduct, aldehydes, ketone, sulfhydryl, haloacetyl, alkyl halide, alkyl sulfonate, NR<sub>1</sub>R<sub>2</sub> wherein R<sub>1</sub>R<sub>2</sub> is –C(=O)CH=CHC(=O) (maleimide),

thioether, cyano, sugar (such as mannose, galactose, and glucose),  $\alpha,\beta$ -unsaturated carbonyl, alkyl mercurial,  $\alpha,\beta$ -unsaturated sulfone.

**[0214]** In one embodiment, a spacer may comprise one or more abasic nucleotides (i.e., lacking a nucleotide base, but having the sugar and phosphate portions). Exemplary abasic nucleotides include 1'2'-dideoxyribose, 1'-deoxyribose, 1'-deoxarabinose and polymers thereof.

**[0215]** Spacers can comprise heteromeric or homomeric oligomers and polymers of the nonnucleic acid components described herein (e.g., linked by a phosphodiester or phosphorothioate linkage or, alternatively an amide, ester, ether, thioether, disulfide, phosphoramidate, phosphotriester, phosphorodithioate, methyl phosphonate or other linkage). For example, in one embodiment, the spacer moiety comprises a branched spacer component (e.g., glycerol) conjugated via a phosphodiester or phosphorothioate linkage to an oligoethylene glycol such as HEG (see, e.g., C-94). Another example, is a spacer comprising a multivalent spacer component conjugated to an oligoethylene glycol such as HEG.

**[0216]** Other suitable spacers comprise substituted alkyl, substituted polyglycol, optionally substituted polyamine, optionally substituted polyalcohol, optionally substituted polyamide, optionally substituted polyether, optionally substituted polyimine, optionally substituted polyphosphodiester (such as poly(1-phospho-3-propanol)), and the like. Optional substituents include alcohol, alkoxy (such as methoxy, ethoxy, and propoxy), straight or branched chain alkyl (such as C1-C12 alkyl, preferably C1-C12 alkyl), amine, aminoalkyl (such as amino C1-C12 alkyl, preferably C1-C12 alkyl), phosphoramidite, phosphate, thiophosphate, hydrazide, hydrazine, halogen, (such as F, Cl, Br, or I), amide, alkylamide (such as amide C1-C12 alkyl or C1-C12 alkyl), carboxylic acid, carboxylic ester, carboxylic anhydride, carboxylic acid halide, ether, sulfonyl halide, imidate ester, isocyanate, isothiocyanate, haloformate, carbodiimide adduct, aldehydes, ketone, sulphydryl, haloacetyl, alkyl halide, alkyl sulfonate, NR<sub>1</sub>R<sub>2</sub> wherein R<sub>1</sub>R<sub>2</sub> is –

C(=O)CH=CHC(=O) (maleimide), thioether, cyano, sugar (such as mannose, galactose, and glucose),  $\alpha,\beta$ -unsaturated carbonyl, alkyl mercurial,  $\alpha,\beta$ -unsaturated sulfone.

[0217] Other suitable spacers may comprise polycyclic molecules, such as those containing phenyl or cyclohexyl rings. The spacer may be a polyether such as polyphosphopropanediol, polyethylene glycol, polypropylene glycol, a bifunctional polycyclic molecule such as a bifunctional pentalene, indene, naphthalene, azulene, heptalene, biphenylene, asymindacene, sym-indacene, acenaphthylene, fluorene, phenalene, phenanthrene, anthracene, fluoranthene, acephenathrylene, aceanthrylene, triphenylene, pyrene, chrysene, naphthacene, thianthrene, isobenzofuran, chromene, xanthene, phenoxathiin, which may be substituted or modified, or a combination of the polyethers and the polycyclic molecules. The polycyclic molecule may be substituted or polysubstituted with C1-C5 alkyl, C6 alkyl, alkenyl, hydroxyalkyl, halogen or haloalkyl group. Nitrogen-containing polyheterocyclic molecules (*e.g.*, indolizine) are typically not suitable spacers. The spacer may also be a polyalcohol, such as glycerol or pentaerythritol. In one embodiment, the spacer comprises (1-phosphopropane)<sub>3</sub>-phosphate or (1-phosphopropane)<sub>4</sub>-phosphate (also called tetraphosphopropanediol and pentaphosphopropanediol). In one embodiment, the spacer comprises derivatized 2,2'-ethylenedioxydiethylamine (EDDA).

[0218] Other examples of non-nucleic acid spacers that may be used in CICs include "linkers" described by Cload and Schepartz, *J. Am. Chem. Soc.* (1991), 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* (1991), 113:5109; Ma et al., *Nucleic Acids Research* (1993), 21:2585; Ma et al., *Biochemistry* (1993), 32:1751; McCurdy et al., *Nucleosides & Nucleotides* (1991), 10:287; Jaschke et al., *Tetrahedron Lett.* (1993), 34:301; Ono et al., *Biochemistry* (1991), 30:9914; and Arnold et al., International Publication No. WO 89/02439 and EP0313219B1 entitled "Non-nucleic acid Linking Reagents for Nucleotide Probes," linkers described by Salunkhe et al., *J. Am. Chem. Soc.* (1992), 114:8768; Nelson et al., *Biochemistry*

35:5339-5344 (1996); Bartley et al., *Biochemistry* 36:14502-511 (1997); Dagneaux et al. *Nucleic Acids Research* 24:4506-12 (1996); Durand et al., *Nucleic Acids Research* 18:6353-59 (1990); Reynolds et al., *Nucleic Acids Research*, 24:760-65 (1996); Hendry et al. *Biochemica et Biophysica Acta*, 1219:405-12 (1994); Altmann et al., *Nucleic Acids Research*, 23:4827-35 (1995), and U.S. Patent No. 6,117,657 (Usman et al.).

[0219] Suitable spacer moieties can contribute charge and/or hydrophobicity to the CIC, contribute favorable pharmacokinetic properties (e.g., improved stability, longer residence time in blood) to the CIC, and/or result in targeting of the CIC to particular cells or organs. Spacer moieties can be selected or modified to tailor the CIC for desired pharmacokinetic properties, induction of a particular immune response, or suitability for desired modes of administration (e.g., oral administration).

[0220] In a CIC comprising more than one spacer moiety, the spacers may be the same or different. Thus, in one embodiment all of the non-nucleic acid spacer moieties in a CIC have the same structure. In one embodiment, a CIC comprises non-nucleic acid spacer moieties with at least 2, at least 3, at least 4, at least 5, or at least 6 or more different structures.

[0221] In some contemplated embodiments of the invention, the spacer moiety of a CIC is defined to exclude certain structures. Thus, in some embodiments of the invention, a spacer is other than an abasic nucleotide or polymer of abasic nucleotides. In some embodiments of the invention, a spacer is other than a oligo(ethyleneglycol) (e.g., HEG, TEG and the like) or poly(ethyleneglycol). In some embodiments a spacer is other than a C3 alkyl spacer. In some embodiments a spacer is other than an alkyl or substituted spacer. In some embodiments, a spacer is other than a polypeptide. Thus, in some embodiments, an immunogenic molecule, e.g., a protein or polypeptide, is not suitable as a component of spacer moieties. However, as discussed *infra*, it is contemplated that in certain embodiments, a CIC is a "proteinaceous CIC" i.e., comprising a spacer moiety comprising a polypeptide

(i.e., oligomer or polymer of amino acids). For example, as discussed *infra*, in some embodiments, a polypeptide antigen can be used as a platform (multivalent spacer) to which a plurality of nucleic acid moieties are conjugated. However, in some embodiments, the spacer moiety is not proteinaceous and/or is not an antigen (i.e., the spacer moiety, if isolated from the CIC, is not an antigen).

[0222] Suitable spacer moieties do not render the CIC of which they are a component insoluble in an aqueous solution (e.g., PBS, pH 7.0). Thus, the definition of spacers excludes microcarriers or nanocarriers. In addition, a spacer moiety that has low solubility, such as a dodecyl spacer (solubility < 5mg/ml when measured as dialcohol precursor 1,12-dihydroxydodecane) is not preferred because it can reduce the hydrophilicity and activity of the CIC. Preferably, spacer moieties have solubility much greater than 5 mg/ml (e.g., solubility at least about 20 mg/ml, at least about 50 mg/ml or at least about 100 mg/ml), e.g., when measured as dialcohol precursors. The form of the spacer moiety used for testing its water solubility is generally its most closely related unactivated and unprotected spacer precursor molecule. For example, C-19 contains a spacer moiety including a dodecyl group with phosphorothioate diester linkages at the C-1 and C-12 positions, thereby connecting the spacer moiety to the nucleic acid moieties. In this case, the water solubility of the dialcohol version of the dodecyl spacer, 1,12-dihydroxydodecane, was tested and found to be less than 5 mg/ml. Spacers with higher water solubility, when tested as their dialcohol precursors, resulted in more immunostimulatory CICs. These higher water solubility spacers include, without limitation, propane 1,3 diol; glycerol; butane-1,4-diol; pentane-1,5-diol; hexane-1,6-diol; triethylene glycol, tetraethylene glycol and HEG.

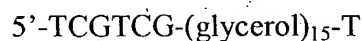
#### A. Charged and Multiunit Spacer Moieties

[0223] The charge of a CIC may be contributed by phosphate, thiophosphate, or other groups in the nucleic acid moieties as well as groups in non-nucleic acid spacer

moieties. In some embodiments of the invention, a non-nucleic acid spacer moiety carries a net charge (e.g., a net positive charge or net negative charge when measured at pH 7). In one embodiment, the CIC has a net negative charge. In some embodiments, the negative charge of a spacer moiety in a CIC is increased by derivatizing a spacer subunit described herein to increase its charge. For example, glycerol can be covalently bound to two nucleic acid moieties and the remaining alcohol can be reacted with an activated phosphoramidite, followed by oxidation or sulfurization to form a phosphate or thiophosphate, respectively. In certain embodiments the negative charge contributed by the non-nucleic acid spacer moieties in a CIC (i.e., the sum of the charges when there is more than one spacer) is greater than the negative charge contributed by the nucleic acid moieties of the CIC. Charge can be calculated based on molecular formula, or determined experimentally, e.g., by capillary electrophoresis (Li, ed., 1992, *Capillary Electrophoresis, Principles, Practice and Application* Elsevier Science Publishers, Amsterdam, The Netherlands, pp202-206).

[0224] As is noted *supra*, suitable spacers include polymers of smaller non-nucleic acid (e.g., non-nucleotide) compounds that may be used as spacers. The smaller non-nucleic acid compounds include compounds commonly referred to as non-nucleotide “linkers” and other spacers described herein. Such polymers (i.e., “multiunit spacers”) may be heteromeric or homomeric, and often comprise monomeric units (e.g., oligoethylene glycols, [e.g.,  $\text{HO}-(\text{CH}_2\text{CH}_2\text{-O})_N\text{-H}$ , where  $N=2-10$ ; such as HEG and TEG], glycerol, 1'2'-dideoxyribose, and the like) linked by an ester linkage (e.g., phosphodiester or phosphorothioate ester). Thus, in one embodiment the spacer comprises a polymeric (e.g., heteropolymeric) structure of non-nucleotide units (e.g., from 2 to about 100 units, alternatively 2 to about 50, e.g., 2 to about 5, alternatively e.g., about 5 to about 50, e.g., about 5 to about 20).

[0225] For illustration, CICs containing multiunit spacers include



where (C3)<sub>15</sub> means 15 propyl linkers connected via phosphorothioate esters; (glycerol)<sub>15</sub> means 15 glycerol linkers connected via phosphorothioate esters; (TEG)<sub>8</sub> means 8 triethyleneglycol linkers connected via phosphorothioate esters; and (HEG)<sub>4</sub> means 4 hexaethyleneglycol linkers connected via phosphorothioate esters. It will be appreciated that certain multiunit spacers have a net-negative charge, and that the negative charge can be increased by increasing the number of *e.g.*, ester-linked monomeric units.

#### B. Multivalent Spacer Moiety

[0226] In certain embodiments, a spacer moiety is a multivalent non-nucleic acid spacer moiety (*i.e.*, a “multivalent spacer”). As used in this context, a CIC containing a multivalent spacer contains a spacer covalently bound to three (3) or more nucleic acid moieties. Multivalent spacers are sometimes referred to in the art as “platform molecules.” Multivalent spacers can be polymeric or nonpolymeric. Examples of suitable molecules include glycerol or substituted glycerol (*e.g.*, 2-hydroxymethyl glycerol, levulinyglycerol); tetraaminobenzene, heptaaminobetacyclodextrin, 1,3,5-trihydroxycyclohexane, pentaerythritol and derivatives of pentaerythritol, tetraaminopentaerythritol, 1,4,8,11-tetraazacyclo tetradecane (Cyclam), 1,4,7,10-tetraazacyclododecane (Cyclen), polyethyleneimine, 1,3-diamino-2-propanol and substituted derivatives (*e.g.*, “symetrical doubler”), [propyloxymethyl]ethyl compounds (*e.g.*, “trebler”), polyethylene glycol derivatives such as so-called “Star PEGs” and “bPEG” (see, *e.g.*, Gnanou et al., 1988, *Makromol. Chem.* 189:2885; Rein et al., 1993, *Acta Polymer* 44:225, Merrill et al., U.S. pat. no.

5,171,264; Shearwater Polymers Inc., Huntsville AL), dendrimers and polysaccharides.

**[0227]** Dendrimers are known in the art and are chemically defined globular molecules, generally prepared by stepwise or reiterative reaction of multifunctional monomers to obtain a branched structure (see, *e.g.*, Tomalia et al., 1990, *Angew. Chem. Int. Ed. Engl.* 29:138-75). A variety of dendrimers are known, *e.g.*, amine-terminated polyamidoamine, polyethyleneimine and polypropyleneimine dendrimers. Exemplary dendrimers for use in the present invention include "dense star" polymers or "starburst" polymers such as those described in U. S. Pat. Nos. 4,587,329; 5,338,532; and 6,177,414, including so-called "poly(amidoamine) ("PAMAM") dendrimers." Still other multimeric spacer molecules suitable for use within the present invention include chemically-defined, non-polymeric valency platform molecules such as those disclosed in U.S. patent 5,552,391; and PCT applications PCT/US00/15968 (published as WO 00/75105); PCT/US96/09976 (published as WO 96/40197), PCT/US97/10075 (published as WO 97/46251); PCT/US94/10031 (published as WO 95/07073); and PCT/US99/29339 (published as WO 00/34231). Many other suitable multivalent spacers can be used and will be known to those of skill in the art.

**[0228]** Conjugation of a nucleic acid moiety to a platform molecule can be effected in any number of ways, typically involving one or more crosslinking agents and functional groups on the nucleic acid moiety and platform molecule. Linking groups are added to platforms using standard synthetic chemistry techniques. Linking groups can be added to nucleic acid moieties using standard synthetic techniques.

**[0229]** Multivalent spacers with a variety of valencies may be used in the practice of the invention, and in various embodiments the multivalent spacer of a CIC is bound to between about 3 and about 400 nucleic acid moieties, sometimes about 100 to about 500, sometimes about 150 to about 250, sometimes 3-200, sometimes from



3 to 100, sometimes from 3-50, frequently from 3-10, and sometimes more than 400 nucleic acid moieties. In various embodiments, the multivalent spacer is conjugated to more than 10, more than 25, more than 50, more than 100 or more than 500 nucleic acid moieties (which may be the same or different). It will be appreciated that, in certain embodiments in which a CIC comprises a multivalent spacer, the invention provides a population of CICs with slightly different molecular structures. For example, when a CIC is prepared using a dendrimer, polysaccharide or other multivalent spacer with a high valency, a somewhat heterogeneous mixture of molecules is produced, i.e., comprising different numbers (within or predominantly within a determinable range) of nucleic acid moieties joined to the multivalent spacer moiety. When a dendrimer, polysaccharide or the like is used as an element of a multivalent spacer, the nucleic acid moieties can be joined directly or indirectly to the element (e.g., dendrimer). For example, a CIC can comprise nucleic acid moiety joined to a dendrimer via an oligoethyleneglycol element (where the dendrimer + oligoethyleneglycol constitute the spacer moiety). It will be recognized that the nucleic acid moieties may be conjugated to more than one spacer moiety, as described in § III(1)B, *supra*.

[0230] Polysaccharides derivitized to allow linking to nucleic acid moieties can be used as multivalent spacers in CICs. Suitable polysaccharides may be naturally occurring polysaccharides or synthetic polysaccharides. Exemplary polysaccharides include, e.g., dextran, mannin, chitosan, agarose, and starch. Mannin may be used, for example, because there are mannin (mannose) receptors on immunologically relevant cell types, such as monocytes and alveolar macrophages, and so the polysaccharide spacer moiety may be used for targeting particular cell types. In an embodiment, the polysaccharide is cross-linked. One suitable compound is epichlorohydrin-crosslinked sucrose (e.g., FICOLL®). FICOLL® is synthesized by cross-linking sucrose with epichlorohydrin which results in a highly branched structure. For example, as shown in Example 49, aminoethylcarboxymethyl-ficoll

(AECM-Ficoll) can be prepared by the method of Inman, 1975, *J. Imm.* 114:704-709. The number of nucleic acid moieties in a CIC comprising a polysaccharide can be any range described herein for a CIC (e.g., a multivalent CIC). For example, in one embodiment, the polysaccharide comprises between about 150 and about 250 nucleic acid moieties. AECM-Ficoll can then be reacted with a heterobifunctional crosslinking reagent, such as 6-maleimido caproic acyl N-hydroxysuccinimide ester, and then conjugated to a thiol-derivatized nucleic acid moiety (see Lee, et al., 1980, *Mol. Imm.* 17:749-56). Other polysaccharides may be modified similarly.

### 5. Synthesis of CICs

[0231] It will be well within the ability of one of skill, guided by this specification and knowledge in the art, to prepare CICs using routine methods. Techniques for making nucleic acid moieties (e.g., oligonucleotides and modified oligonucleotides) are known. Nucleic acid moieties can be synthesized using techniques including, but not limited to, enzymatic methods and chemical methods and combinations of enzymatic and chemical approaches. For example, DNA or RNA containing phosphodiester linkages can be chemically synthesized by sequentially coupling the appropriate nucleoside phosphoramidite to the 5'-hydroxy group of the growing oligonucleotide attached to a solid support at the 3'-end, followed by oxidation of the intermediate phosphite triester to a phosphate triester. Useful solid supports for DNA synthesis include Controlled Pore Glass (Applied Biosystems, Foster City, CA), polystyrene bead matrix (Primer Support, Amersham Pharmacia, Piscataway, NJ) and TentGel (Rapp Polymere GmbH, Tübingen, Germany). Once the desired oligonucleotide sequence has been synthesized, the oligonucleotide is removed from the support, the phosphate triester groups are deprotected to phosphate diesters and the nucleoside bases are deprotected using aqueous ammonia or other bases.

**[0232]** For instance, DNA or RNA polynucleotides (nucleic acid moieties) containing phosphodiester linkages are generally synthesized by repetitive iterations of the following steps: a) removal of the protecting group from the 5'-hydroxyl group of the 3'-solid support-bound nucleoside or nucleic acid, b) coupling of the activated nucleoside phosphoramidite to the 5'-hydroxyl group, c) oxidation of the phosphite triester to the phosphate triester, and d) capping of unreacted 5'-hydroxyl groups. DNA or RNA containing phosphorothioate linkages is prepared as described above, except that the oxidation step is replaced with a sulfurization step. Once the desired oligonucleotide sequence has been synthesized, the oligonucleotide is removed from the support, the phosphate triester groups are deprotected to phosphate diesters and the nucleoside bases are deprotected using aqueous ammonia or other bases. See, for example, Beaucage (1993) "Oligodeoxyribonucleotide Synthesis" in *PROTOCOLS FOR OLIGONUCLEOTIDES AND ANALOGS, SYNTHESIS AND PROPERTIES* (Agrawal, ed.) Humana Press, Totowa, NJ; Warner et al. (1984) *DNA* 3:401; Tang et al. (2000) *Org. Process Res. Dev.* 4:194-198; Wyrzykiewica et al. (1994) *Bioorg. & Med. Chem. Lett.* 4:1519-1522; Radhakrishna et al. (1989) *J. Org. Chem.* 55:4693-4699, and U.S. Patent No. 4,458,066. Programmable machines that automatically synthesize nucleic acid moieties of specified sequences are widely available. Examples include the Expedite 8909 automated DNA synthesizer (Perseptive Biosystem, Framington MA); the ABI 394 (Applied Biosystems, Inc., Foster City, CA); and the OligoPilot II (Amersham Pharmacia Biotech, Piscataway, NJ)

**[0233]** Polynucleotides can be assembled in the 3' to 5' direction, e.g., using base-protected nucleosides (monomers) containing an acid-labile 5'-protecting group and a 3'-phosphoramidite. Examples of such monomers include 5'-O-(4,4'-dimethoxytrityl)-protected nucleoside-3'-O-(N,N-diisopropylamino) 2-cyanoethyl phosphoramidite, where examples of the protected nucleosides include, but are not limited to, N6-benzoyladenine, N4-benzoylcytosine, N2-isobutryrylguanosine, thymidine, and uridine. In this case, the solid support used contains a 3'-linked

protected nucleoside. Alternatively, polynucleotides can be assembled in the 5' to 3' direction using base-protected nucleosides containing an acid-labile 3'-protecting group and a 5'-phosphoramidite. Examples of such monomers include 3'-O-(4,4'-dimethoxytrityl)-protected nucleoside-5'-O-(N,N-diisopropylamino) 2-cyanoethyl phosphoramidite, where examples of the protected nucleosides include, but are not limited to, N6-benzoyladenine, N4-benzoylcytosine, N2-isobutryrylguanosine, thymidine, and uridine (Glen Research, Sterling, VA). In this case, the solid support used contains a 5'-linked protected nucleoside. Circular nucleic acid components can be isolated, synthesized through recombinant methods, or chemically synthesized. Chemical synthesis can be performed using any method described in the literature. See, for instance, Gao et al. (1995) *Nucleic Acids Res.* 23:2025-2029 and Wang et al. (1994) *Nucleic Acids Res.* 22:2326-2333.

**[0234]** Conjugation of the nucleic acid moieties and spacer moieties can be carried out in a variety of ways, depending on the particular CIC being prepared. Methods for addition of particular spacer moieties are known in the art and, for example, are described in the references cited *supra*. See, e.g., Durand et al., *Nucleic Acids Research* 18:6353-59 (1990). The covalent linkage between a spacer moiety and nucleic acid moiety can be any of a number of types, including phosphodiester, phosphorothioate, amide, ester, ether, thioether, disulfide, phosphoramidate, phosphotriester, phosphorodithioate, methyl phosphonate and other linkages. As noted *supra*, spacer moiety precursors can optionally be modified with terminal activating groups for coupling to nucleic acids. Examples of activated spacer moieties can be seen in Figure 1 where protecting groups suitable for automated synthesis have been added. Other spacer moiety precursors include, for example and not for limitation, (1)  $\text{HOCH}_2\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CH}_2\text{OH}$ , where  $n=0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44$  or 45 or is greater than 45;

(2)  $\text{HOCH}_2\text{CHOHCH}_2\text{OH}$ ; (3)  $\text{HO}(\text{CH}_2)_n\text{OH}$ , where  $n = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10$ , or 11.

[0235] In one embodiment, a spacer moiety precursor is used that includes first and second reactive groups to permit conjugation to nucleic acid moieties in a stepwise fashion, in which the first reactive group has the property that it can couple efficiently to the terminus of a growing chain of nucleic acids and the second reactive group is capable of further extending, in a step-wise fashion the growing chain of mixed nucleotide and non-nucleotide moieties in the CIC. It will often be convenient to combine a spacer moiety(s) and a nucleic acid moiety(s) using the same phosphoramidite-type chemistry used for synthesis of the nucleic acid moiety. For example, CICs of the invention can be conveniently synthesized using an automated DNA synthesizer (e.g., Expedite 8909; Perseptive Biosystems, Framington, MA) using phosphoramidite chemistry (see, e.g., Beaucage, 1993, *supra*; *Current Protocols in Nucleic Acid Chemistry, supra*). However, one of skill will understand that the same (or equivalent) synthesis steps carried out by an automated DNA synthesizer can also be carried out manually, if desired. The resulting linkage between the nucleic acid and the spacer precursors can be a phosphorothioate or phosphodiester linkage. In such a synthesis, typically, one end of the spacer (or spacer subunit for multimeric spacers) is protected with a 4,4'-dimethoxytrityl group, while the other end contains a phosphoramidite group.

[0236] A variety of spacers with useful protecting and reacting groups are commercially available, for example:

triethylene glycol spacer or "TEG spacer" 9-O-(4,4'-dimethoxytrityl)triethyleneglycol-1-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Glen Research, 22825 Davis Drive, Sterling, VA);

hexaethylene glycol spacer or "HEG spacer" 18-O-(4,4'-dimethoxytrityl)hexaethyleneglycol-1-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Glen Research, Sterling, VA);

propyl spacer 3-(4,4'-dimethoxytrityloxy)propyloxy-1-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Glen Research, Sterling, VA);

butyl spacer 4-(4,4'-dimethoxytrityloxy)butyloxy-1-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Chem Genes Corporation, Ashland Technology Center, 200 Homer Ave, Ashland, MA);

Hexyl spacer: 6-(4,4'-dimethoxytrityloxy)hexyloxy-1-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Biosearch Technologies, Novato, CA)

2-(hydroxymethyl)ethyl spacer or "HME spacer" 1-(4,4'-dimethoxytrityloxy)-3-(levulinyloxy)-propyloxy-2-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite]; also called "asymmetrical branched" spacer (see Fig. 2) (Chem Genes Corp., Ashland Technology Center, Ashland MA.);

"abasic nucleotide spacer" or "abasic spacer" 5-O-(4,4'-dimethoxytrityl)-1,2-dideoxyribose-3-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Glen Research, Sterling, VA);

"symmetrical branched spacer" or "glycerol spacer" 1,3-O,O-bis(4,4'-dimethoxytrityl)glycerol-2-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Chem Genes, Ashland, MA) (see Fig. 2);

"trebler spacer" (see Fig. 2) 2,2,2-O,O,O-tris[3-O-(4,4'-dimethoxytrityloxy)propyloxymethyl]ethyl-1-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Glen Research, Sterling, VA);

"symmetrical doubler spacer" (see Fig. 2) 1,3-O,O-bis[5-O-(4,4'-dimethoxytrityloxy)pentylamido]propyl-2-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Glen Research, Sterling, VA);

"dodecyl spacer" 12-(4,4'-dimethoxytrityloxy)dodecyloxy-1-O-[(2-cyanoethyl) N,N-diisopropylphosphoramidite] (Glen Research, Sterling, VA).

[0237] These and a large variety of other protected spacer moiety precursors (e.g., comprising DMT and phosphoramidite group protecting groups) can be purchased or can be synthesized using routine methods for use in preparing CICs disclosed herein.

The instrument is programmed according to the manufacturer's instructions to add nucleotide monomers and spacers in the desired order.

**[0238]** CICs prepared "in situ" on a DNA synthesizer require protected nucleoside and protected spacer monomers, both containing reactive or activatable functional groups. The reactive and/or protected form of the spacer moiety can be described as a "spacer precursor component." It will be appreciated by those with skill in the art that the reactive groups in the spacer precursors form stable linkages after coupling and the protecting groups on the spacer precursor are removed in the resultant spacer moiety in the CIC. The protecting groups are generally removed during the step-wise synthesis of the CIC, in order to allow reaction at that site. In cases where there are protecting groups on additional reactive groups, the protecting groups are removed after the step-wise synthesis of the CIC (such as the levulinyl group on the spacer precursor shown in Figure 2, structure 3, used to make C-25).

**[0239]** An example of a spacer precursor with no additional reactive functionality is 18-O-(4,4'-dimethoxytrityl)hexaethyleneglycol-1-O-[(2-cyanoethyl)N,N-diisopropylphosphoramidite], which contains a protecting group, the 4,4'-dimethoxytrityl group, and a reactive group, the (2-cyanoethyl)N,N-diisopropylphosphoramidite group. During preparation of the CIC using phosphoramidite chemistry on a DNA synthesizer, the (2-cyanoethyl)N,N-diisopropylphosphoramidite group in the spacer precursor is activated by a weak acid, such as 1H-tetrazole, and reacted with the free 5'-hydroxyl of the nucleobase-protected nucleic acid moiety to form a phosphite triester. The phosphite triester group is then either oxidized or sulfurized to a stable phosphotriester or phosphorothioate triester group, respectively. The resultant triester group is stable to the rest of the CIC synthesis and remains in that form until the final deprotection. In order to couple either another spacer precursor or an activated nucleoside monomer, which will become part of the next nucleic acid moiety, the 4,4'-dimethoxytrityl group on the spacer precursor is removed. After coupling and either oxidation or

sulfurization, this group also becomes either a stable phosphotriester or phosphorothioate triester group, respectively. Once the protected CIC is fully assembled, the CIC is cleaved from the solid support, the cyanoethyl groups on the phosphotriester or phosphorothioate triester groups are removed, and the nucleobase protection is removed. In this example, the CIC contains spacer moieties including stable phosphodiester or phosphorothioate diester linkages to the nucleic acid moieties. Both the reactive phosphoramidite group and the protected hydroxyl group of the spacer precursor are converted to stable phosphodiester or phosphorothioate diester linkages in the spacer moiety. Because the reaction of each end of the spacer may be independent, one linkage may be phosphodiester and the other linkage phosphorothioate diester, or any combination thereof. CICs with other phosphate modifications, such as phosphorodithioate, methyl phosphonate, and phosphoramidate, may also be prepared in this manner by using a spacer precursor with the appropriate reactive group, the correct ancilliary reagents, and protocols designed for that type of linkage. These protocols are analogous to those described for preparing nucleic acid moieties with modified phosphate linkages.

**[0240]** Although use of phosphoramidite chemistry is convenient for the preparation of certain CICs, it will be appreciated that the CICs of the invention are not limited to compounds prepared by any particular method of synthesis or preparation. For example, nucleic acid moieties containing groups not compatible with DNA synthesis and deprotection conditions, such as (but not limited to) hydrazine or maleimide, can be prepared by reacting a nucleic acid moiety containing an amino linker with the appropriate heterobifunctional crosslinking reagent, such as SHNH (succinimidyl hydraziniumnicotinate) or sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate).

**[0241]** Methods for conjugating protein, peptides, oligonucleotides, and small molecules in various combinations are described in the literature and can be adapted to achieve conjugation of a nucleic acid moiety containing a reactive linking group to



a spacer moiety precursor. See, for example, Bioconjugate Techniques, Greg T. Hermanson, Academic Press, Inc., San Diego, CA, 1996. In some embodiments, a nucleic acid moiety(s) is synthesized, and a reactive linking group (*e.g.*, amino, carboxylate, thio, disulfide, and the like) is added using standard synthetic chemistry techniques. The reactive linking group (which is considered to form a portion of the resulting spacer moiety) is conjugated to additional non-nucleic acid compounds (for example, without limitation, a compound listed in §4, *supra*) to form a portion of the spacer moiety. Reactive linking groups are added to nucleic acids using standard methods for nucleic acid synthesis, employing a variety of reagents known in the art. Examples include reagents that contain a protected amino group, carboxylate group, thiol group, disulfide group, aldehyde group, diol group, diene group and a phosphoramidite group. Once these compounds are incorporated into the nucleic acids, via the activated phosphoramidite group, and are deprotected, they provide nucleic acids with amino, carboxylate, aldehyde, diol, diene or thiol reactivity. Examples of reactive groups for conjugating a nucleic acid moiety containing a reactive linker group to a spacer moiety precursor that contains a reactive group are shown below.

<u>nucleic acid reactive</u> <u>group</u>	<u>Spacer moiety precursor</u> <u>reactive group</u>	<u>Stable linkage formed</u>
thiol	maleimide, haloacetyl	thioether
maleimide	thiol	thioether
thiol	pyridine disulfide	disulfide
pyridine disulfide	thiol	disulfide
amine	NHS or other active ester	amide
amine	carboxylate	amide
carboxylate	amine	amide
aldehyde, ketone	hydrazine, hydrazide	hydrazone, hydrazide
hydrazine, hydrazide	aldehyde, ketone	hydrazone, hydrazide
diene	dienophile	aliphatic or heterocyclic ring

[0242] The reactive linking group and the spacer precursor react to form a stable bond and the entire group of atoms between the two (or more) nucleic acid moieties

is defined as the spacer moiety. For example, a nucleic acid moiety synthesized with a mercaptohexyl group linked to the nucleic acid moiety via a phosphorothioate group can be reacted with a spacer precursor containing one (or more) maleimide group(s), forming a thioether linkage(s). The spacer moiety of this CIC includes the phosphorothioate group and hexyl group from the linker on the nucleic acid moiety, the new thioether linkage, and the rest of the spacer that was part of the spacer precursor.

[0243] Although linear CICs can be made using these conjugation strategies, these methods are most often applied for the preparation of branched CICs. Additionally, spacer precursor molecules can be prepared with several orthogonal reactive groups to allow for the addition of more than one type nucleic acid moiety (e.g., different sequence motif).

[0244] In one embodiment, CICs with multivalent spacers conjugated to more than one type of nucleic acid moiety are prepared. For instance, platforms containing two maleimide groups (which can react with thiol-containing polynucleotides), and two activated ester groups (which can react with amino-containing nucleic acids) have been described (see, e.g., PCT/US94/10031, published as WO 95/07073). These two activated groups can be reacted independently of each other. This would result in a CIC containing a total of 4 nucleic acid moieties, two of each sequence.

[0245] CICs with multivalent spacers containing two different nucleic acid sequences can also be prepared using the symmetrical branched spacer, described above, and conventional phosphoramidite chemistry (e.g., using manual or automated methods). The symmetrical branched spacer contains a phosphoramidite group and two protecting groups that are the same and are removed simultaneously. In one approach, for example, a first nucleic acid is synthesized and coupled to the symmetrical branched spacer, the protecting groups are removed from the spacer. Then two additional nucleic acids (of the same sequence) are synthesized on the

spacer (using double the amount of reagents used for synthesis of a single nucleic acid moiety in each step). This procedure is described in detail in Example 15, *infra*.

[0246] A similar method can be used to connect three different nucleic acid moieties (referred to below as Nucleic acids I, II, and III) to a multivalent platform (e.g., asymmetrical branched spacer). This is most conveniently carried out using an automated DNA synthesizer. In one embodiment, the asymmetrical branched spacer contains a phosphoramidite group and two orthogonal protecting groups that can be removed independently. First, nucleic acid I is synthesized, then the asymmetrical branched spacer is coupled to nucleic acid I, then nucleic acid II is added after the selective removal of one of the protecting groups. Nucleic acid II is deprotected, and capped, and then the other protecting group on the spacer is removed. Finally, nucleic acid III is synthesized. This procedure is described in detail in Example 17, *infra*.

[0247] Hydrophilic linkers of variable lengths are may be used, for example to link nucleic acids moieties and platform molecules. A variety of suitable linkers are known. Suitable linkers include, without limitation, linear oligomers or polymers of ethylene glycol. Such linkers include linkers with the formula  $R^1S(CH_2CH_2O)_nCH_2CH_2O(CH_2)_mCO_2R^2$  wherein  $n = 0-200$ ,  $m = 1$  or  $2$ ,  $R^1 = H$  or a protecting group such as trityl,  $R^2 = H$  or alkyl or aryl, e.g., 4-nitrophenyl ester. These linkers may be used in connecting a molecule containing a thiol reactive group such as haloacetyl, maleimide, etc., via a thioether to a second molecule which contains an amino group via an amide bond. The order of attachment can vary, *i.e.*, the thioether bond can be formed before or after the amide bond is formed. Other linkers include Sulfo-SMCC (sulfosuccinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate) Pierce Chemical Co. product 22322; Sulfo-EMCS (*N*-[ $\epsilon$ -maleimidocaproyloxy] sulfosuccinimide ester) Pierce Chemical Co. product 22307; Sulfo-GMBS (*N*-[ $\gamma$ -maleimidobutyryloxy] sulfosuccinimide ester) Pierce Chemical Co. product 22324 (Pierce Chemical Company, Rockford, IL), and similar

compounds of the general formula-maleimido-R-C(O)NHS ester, where R = alkyl, cyclic alkyl, polymers of ethylene glycol, and the like.

#### 6. Proteinaceous CICs

[0248] In certain embodiments, a polypeptide, such as a protein antigen or antigen fragment, is used as a multivalent spacer moiety to which a plurality of nucleic acid moieties are covalently conjugated, directly or via linkers, to form a "proteinaceous CIC." The polypeptide can be an antigen or immunogen to which an adaptive immune response is desired, or a carrier (*e.g.*, albumin). Typically, a proteinaceous CIC comprises at least one, and usually several or many nucleic acid moieties that (a) are between 2 and 7, more often between 4 and 7 nucleotides in length, alternatively between 2 and 6, 2 and 5, 4 and 6, or 4 and 5 nucleotides in length and/or (b) have inferior isolated immunomodulatory activity or do not have isolated immunomodulatory activity. Methods of making a proteinaceous CIC will be apparent to one of skill upon review of the present disclosure. A nucleic acid, for example, can be covalently conjugated to a polypeptide spacer moiety by art known methods including linkages between a 3' or 5' end of a nucleic acid moiety (or at a suitably modified base at an internal position in the a nucleic acid moiety) and a polypeptide with a suitable reactive group (*e.g.*, an N-hydroxysuccinimide ester, which can be reacted directly with the N<sup>4</sup> amino group of cytosine residues). As a further example, a polypeptide can be attached to a free 5'-end of a nucleic acid moiety through an amine, thiol, or carboxyl group that has been incorporated into nucleic acid moiety. Alternatively, the polypeptide can be conjugated to a spacer moiety, as described herein. Further, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite can be covalently attached to a hydroxyl group of a polynucleotide, and, subsequent to deprotection, the functionality can be used to covalently attach the CIC to a peptide.

## 7. Purification

[0249] The CICs of the invention are purified using any conventional means, such as high performance liquid chromatography, electrophoretic methods, nucleic acid affinity chromatography, size exclusion chromatography, and ion exchange chromatography. In some embodiments, a CIC is substantially pure, *e.g.*, at least about 80% pure by weight, often at least about 90% pure by weight, more often at least about 95% pure, most often at least about 85% pure.

## 8. Compositions

[0250] In various embodiments, compositions of the invention comprise one or more CICs, (i.e. a single CIC or a combination of two or more CICs) optionally in conjunction with another immunomodulatory agent, such as a peptide, an antigen (described below) and/or an additional adjuvant. Compositions of the invention may comprise a CIC and pharmaceutically acceptable excipient. By "pharmaceutically acceptable" it is meant the carrier, diluent or excipient must be compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Pharmaceutically acceptable excipients are well known in the art and include sterile water, isotonic solutions such as saline and phosphate buffered saline, and other excipients known in the art. See, *e.g.*, *Remington: The Science and Practice of Pharmacy (19th edition, 1995, Gennavo, ed.)*. Adjuvants (an example of which is alum) are known in the art. CIC formulations may be prepared with other immunotherapeutic agents, such as cytokines and antibodies. In some embodiments the composition is isotonic and/or sterile, *e.g.*, suitable for administration to a human patient, *e.g.*, manufactured or formulated under GMP standards.

#### A. CIC/MC Complexes

[0251] CICs may be administered in the form of CIC/microcarrier (CIC/MC) complexes. Accordingly, the invention provides compositions comprising CIC/MC complexes.

[0252] CIC/MC complexes comprise a CIC bound to the surface of a microcarrier (*i.e.*, the CIC is not encapsulated in the MC), and preferably comprise multiple molecules of CIC bound to each microcarrier. In certain embodiments, a mixture of different CICs may be complexed with a microcarrier, such that the microcarrier is bound to more than one CIC species. The bond between the CIC and MC may be covalent or non-covalent (*e.g.* mediated by ionic and/or hydrophobic interactions).

As will be understood by one of skill in the art, the CIC may be modified or derivatized and the composition of the microcarrier may be selected and/or modified to accommodate the desired type of binding desired for CIC/MC complex formation.

[0253] Covalently bonded CIC/MC complexes may be linked using any covalent crosslinking technology known in the art. Typically, the CIC portion will be modified, either to incorporate an additional moiety (*e.g.*, a free amine, carboxyl or sulfhydryl group) or incorporate modified (*e.g.*, phosphorothioate) nucleotide bases to provide a site at which the CIC portion may be linked to the microcarrier. The link between the CIC and MC portions of the complex can be made at the 3' or 5' end of the CIC, or at a suitably modified base at an internal position in the CIC. The microcarrier is generally also modified to incorporate moieties through which a covalent link may be formed, although functional groups normally present on the microcarrier may also be utilized. The CIC/MC is formed by incubating the CIC with a microcarrier under conditions which permit the formation of a covalent complex (*e.g.*, in the presence of a crosslinking agent or by use of an activated microcarrier comprising an activated moiety which will form a covalent bond with the CIC).

[0254] A wide variety of crosslinking technologies are known in the art, and include crosslinkers reactive with amino, carboxyl and sulfhydryl groups. As will be apparent to one of skill in the art, the selection of a crosslinking agent and crosslinking protocol will depend on the configuration of the CIC and the microcarrier as well as the desired final configuration of the CIC/MC complex. The crosslinker may be either homobifunctional or heterobifunctional. When a homobifunctional crosslinker is used, the crosslinker exploits the same moiety on the CIC and MC (*e.g.*, an aldehyde crosslinker may be used to covalently link a CIC and MC where both the CIC and MC comprise one or more free amines). Heterobifunctional crosslinkers utilize different moieties on the CIC and MC, (*e.g.*, a maleimido-N-hydroxysuccinimide ester may be used to covalently link a free sulfhydryl on the CIC and a free amine on the MC), and are preferred to minimize formation of inter-microcarrier bonds. In most cases, it is preferable to crosslink through a first crosslinking moiety on the microcarrier and a second crosslinking moiety on the CIC, where the second crosslinking moiety is not present on the microcarrier. One preferred method of producing the CIC/MC complex is by 'activating' the microcarrier by incubating with a heterobifunctional crosslinking agent, then forming the CIC/MC complex by incubating the CIC and activated MC under conditions appropriate for reaction. The crosslinker may incorporate a "spacer" arm between the reactive moieties, or the two reactive moieties in the crosslinker may be directly linked.

[0255] In one preferred embodiment, the CIC portion comprises at least one free sulfhydryl (*e.g.*, provided by a 5'-thiol modified base or linker) for crosslinking to the microcarrier, while the microcarrier comprises free amine groups. A heterobifunctional crosslinker reactive with these two groups (*e.g.*, a crosslinker comprising a maleimide group and a NHS-ester), such as succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate is used to activate the MC, then covalently crosslink the CIC to form the CIC/MC complex.

[0256] Non-covalent CIC/MC complexes may be linked by any non-covalent binding or interaction, including ionic (electrostatic) bonds, hydrophobic interactions, hydrogen bonds, van der Waals attractions, or a combination of two or more different interactions, as is normally the case when a binding pair is to link the CIC and MC.

[0257] Preferred non-covalent CIC/MC complexes are typically complexed by hydrophobic or electrostatic (ionic) interactions, or a combination thereof, (e.g., through base pairing between a CIC and a polynucleotide bound to an MC). Due to the hydrophilic nature of the backbone of polynucleotides, CIC/MC complexes which rely on hydrophobic interactions to form the complex generally require modification of the CIC portion of the complex to incorporate a highly hydrophobic moiety. Preferably, the hydrophobic moiety is biocompatible, nonimmunogenic, and is naturally occurring in the individual for whom the composition is intended (e.g., is found in mammals, particularly humans). Examples of preferred hydrophobic moieties include lipids, steroids, sterols such as cholesterol, and terpenes. The method of linking the hydrophobic moiety to the CIC will, of course, depend on the configuration of the CIC and the identity of the hydrophobic moiety. The hydrophobic moiety may be added at any convenient site in the CIC, preferably at either the 5' or 3' end; in the case of addition of a cholesterol moiety to a CIC, the cholesterol moiety is preferably added to the 5' end of the CIC, using conventional chemical reactions (see, for example, Godard et al. (1995) *Eur. J. Biochem.* 232:404-410). Preferably, microcarriers for use in CIC/MC complexes linked by hydrophobic bonding are made from hydrophobic materials, such as oil droplets or hydrophobic polymers, although hydrophilic materials modified to incorporate hydrophobic moieties may be utilized as well. When the microcarrier is a liposome or other liquid phase microcarrier comprising a lumen, the CIC/MC complex is formed by mixing the CIC and the MC after preparation of the MC, in order to avoid encapsulation of the CIC during the MC preparation process.



[0258] Non-covalent CIC/MC complexes bound by electrostatic binding typically exploit the highly negative charge of the polynucleotide backbone. Accordingly, microcarriers for use in non-covalently bound CIC/MC complexes are generally positively charged at physiological pH (e.g., about pH 6.8-7.4). The microcarrier may intrinsically possess a positive charge, but microcarriers made from compounds not normally possessing a positive charge may be derivatized or otherwise modified to become positively charged. For example, the polymer used to make the microcarrier may be derivatized to add positively charged groups, such as primary amines. Alternately, positively charged compounds may be incorporated in the formulation of the microcarrier during manufacture (e.g., positively charged surfactants may be used during the manufacture of poly(lactic acid)/poly(glycolic acid) copolymers to confer a positive charge on the resulting microcarrier particles). See, e.g., Examples 28 and 34, *infra*.

[0259] Non-covalent CIC/MC complexes linked by nucleotide base pairing may be produced using conventional methodologies. Generally, base-paired CIC/MC complexes are produced using a microcarrier comprising a bound, preferably a covalently bound, polynucleotide (the "capture polynucleotide") that is at least partially complementary to the CIC. The segment of complementarity between the CIC and the capture nucleotide is preferably at least 6, 8, 10 or 15 contiguous base pairs, more preferably at least 20 contiguous base pairs. The capture nucleotide may be bound to the MC by any method known in the art, and is preferably covalently bound to the CIC at the 5' or 3' end.

[0260] In other embodiments, a binding pair may be used to link the CIC and MC in a CIC/MC complex. The binding pair may be a receptor and ligand, an antibody and antigen (or epitope), or any other binding pair which binds at high affinity (e.g.,  $K_d$  less than about  $10^{-8}$ ). One type of preferred binding pair is biotin and streptavidin or biotin and avidin, which form very tight complexes. When using a binding pair to mediate CIC/MC complex binding, the CIC is derivatized, typically by a covalent

linkage, with one member of the binding pair, and the MC is derivatized with the other member of the binding pair. Mixture of the two derivatized compounds results in CIC/MC complex formation.

[0261] Many CIC/MC complex embodiments do not include an antigen, and certain embodiments exclude antigen(s) associated with the disease or disorder which is the object of the CIC/MC complex therapy. In further embodiments, the CIC is also bound to one or more antigen molecules. Antigen may be coupled with the CIC portion of a CIC/MC complex in a variety of ways, including covalent and/or non-covalent interactions. Alternately, the antigen may be linked to the microcarrier. The link between the antigen and the CIC in CIC/MC complexes comprising an antigen bound to the CIC can be made by techniques described herein and known in the art.

#### B. Co-Administered Antigen

[0262] In some embodiments, the CIC is coadministered with an antigen. Any antigen may be co-administered with a CIC and/or used for preparation of compositions comprising a CIC and antigen.

[0263] In some embodiments, the antigen is an allergen. Examples of recombinant allergens are provided in Table 1. Preparation of many allergens is well-known in the art, including, but not limited to, preparation of ragweed pollen allergen Antigen E (Amb a1) (Rafnar et al. (1991) *J. Biol. Chem.* 266:1229-1236), grass allergen Lol p 1 (Tamborini et al. (1997) *Eur. J. Biochem.* 249:886-894), major dust mite allergens Der pI and Der PII (Chua et al. (1988) *J. Exp. Med.* 167:175-182; Chua et al. (1990) *Int. Arch. Allergy Appl. Immunol.* 91:124-129), domestic cat allergen Fel d I (Rogers et al. (1993) *Mol. Immunol.* 30:559-568), white birch pollen Bet vI (Breiteneder et al. (1989) *EMBO J.* 8:1935-1938), Japanese cedar allergens Cry j 1 and Cry j 2 (Kingetsu et al. (2000) *Immunology* 99:625-629), and protein antigens from other tree pollen (Elsayed et al. (1991) *Scand. J. Clin. Lab. Invest.*

Suppl. 204:17-31). Preparation of protein antigens from grass pollen for *in vivo* administration has been reported.

[0264] In some embodiments, the allergen is a food allergen, including, but not limited to, peanut allergen, for example Ara h I (Stanley et al. (1996) *Adv. Exp. Med. Biol.* 409:213-216); walnut allergen, for example, Jug r I (Tueber et al. (1998) *J. Allergy Clin. Immunol.* 101:807-814); brazil nut allergen, for example, albumin (Pastorello et al. (1998) *J. Allergy Clin. Immunol.* 102:1021-1027; shrimp allergen, for example, Pen a I (Reese et al. (1997) *Int. Arch. Allergy Immunol.* 113:240-242); egg allergen, for example, ovomucoid (Crooke et al. (1997) *J. Immunol.* 159:2026-2032); milk allergen, for example, bovine  $\beta$ -lactoglobulin (Selot al. (1999) *Clin. Exp. Allergy* 29:1055-1063); fish allergen, for example, parvalbumins (Van Do et al. (1999) *Scand. J. Immunol.* 50:619-625; Galland et al. (1998) *J. Chromatogr. B. Biomed. Sci. Appl.* 706:63-71). In some embodiments, the allergen is a latex allergen, including but not limited to, Hev b 7 (Sowka et al. (1998) *Eur. J. Biochem.* 255:213-219). Table 1 shows a list of allergens that may be used.

TABLE 1  
RECOMBINANT ALLERGENS

Group	Allergen	Reference
ANIMALS:		
CRUSTACEA		
Shrimp/lobster	tropomyosin Pan s I	Leung et al. (1996) <i>J. Allergy Clin. Immunol.</i> 98:954-961 Leung et al. (1998) <i>Mol. Mar. Biol. Biotechnol.</i> 7:12-20
INSECTS		
Ant	Sol i 2 (venom)	Schmidt et al. <i>J Allergy Clin Immunol.</i> , 1996, 98:82-8
Bee	Phospholipase A2 (PLA)  Hyaluronidase (Hya)	Muller et al. <i>J Allergy Clin Immunol</i> , 1995, 96:395-402 Forster et al. <i>J Allergy Clin Immunol</i> , 1995, 95:1229-35 Muller et al. <i>Clin Exp Allergy</i> , 1997, 27:915-20  Soldatova et al. <i>J Allergy Clin Immunol</i> , 1998, 101:691-8
Cockroach	Bla g Bd9OK  Bla g 4 (a calycin)  Glutathione S-transferase  Per a 3	Helm et al. <i>J Allergy Clin Immunol</i> , 1996, 98:172-180  Vailes et al. <i>J Allergy Clin Immunol</i> , 1998, 101:274-280  Arruda et al. <i>J Biol Chem</i> , 1997, 272:20907-12  Wu et al. <i>Mol Immunol</i> , 1997, 34:1-8

Dust mite	Der p 2 (major allergen)  Der p2 variant Der f2  Der p10 Tyr p 2	Lynch et al. J Allergy Clin Immunol, 1998, 101:562-4 Hakkaart et al. Clin Exp Allergy, 1998, 28:169-74 Hakkaart et al. Clin Exp Allergy, 1998, 28:45-52 Hakkaart et al. Int Arch Allergy Immunol, 1998, 115 (2):150-6 Mueller et al. J Biol Chem, 1997, 272:26893-8  Smith et al. J Allergy Clin Immunol, 1998, 101:423-5  Yasue et al. Clin Exp Immunol, 1998, 113:1-9 Yasue et al. Cell Immunol, 1997, 181:30-7  Asturias et al. Biochim Biophys Acta, 1998, 1397:27-30  Eriksson et al. Eur J Biochem, 1998
Hornet	Antigen 5 aka Dol m V (venom)	Tomalski et al. Arch Insect Biochem Physiol, 1993, 22:303-13
Mosquito	Aed a 1 (salivary apyrase)	Xu et al. Int Arch Allergy Immunol, 1998, 115:245-51
Yellow jacket	antigen 5, hyaluronidase and phospholipase (venom)	King et al. J Allergy Clin Immunol, 1996, 98:588-600
MAMMALS		
Cat	Fel d 1	Slunt et al. J Allergy Clin Immunol, 1995, 95:1221-8 Hoffmann et al. (1997) J Allergy Clin Immunol 99:227-32 Hedlin Curr Opin Pediatr, 1995, 7:676-82
Cow	Bos d 2 (dander; a lipocalin)  $\beta$ -lactoglobulin (BLG, major cow milk allergen)	Zeiler et al. J Allergy Clin Immunol, 1997, 100:721-7 Rautiainen et al. Biochem Bioph. Res Comm., 1998, 247:746-50  Chatel et al. Mol Immunol, 1996, 33:1113-8 Lehrer et al. Crit Rev Food Sci Nutr, 1996, 36:553-64
Dog	Can f 1 and Can f 2, salivary lipocalins	Konieczny et al. Immunology, 1997, 92:577-86 Spitzauer et al. J Allergy Clin Immunol, 1994, 93:614-27 Vrtala et al. J Immunol, 1998, 160:6137-44
Horse	Equ c 1 (major allergen, a lipocalin)	Gregoire et al. J Biol Chem, 1996, 271:32951-9
Mouse	mouse urinary protein (MUP)	Konieczny et al. Immunology, 1997, 92:577-86
OTHER MAMMALIAN ALLERGENS		
Insulin		Ganz et al. J Allergy Clin Immunol, 1990, 86:45-51 Grammer et al. J Lab Clin Med, 1987, 109:141-6 Gonzalo et al. Allergy, 1998, 53:106-7
Interferons	interferon alpha 2c	Detmar et al. Contact Dermatis, 1989, 20:149-50
MOLLUSCS	topomyosin	Leung et al. J Allergy Clin Immunol, 1996, 98:954-61
PLANT ALLERGENS:		
Barley	Hor v 9	Astwood et al. Adv Exp Med Biol, 1996, 409:269-77
Birch	pollen allergen, Bet v 4  rBet v 1 Bet v 2: (profilin)	Twardosz et al. Biochem Bioph. Res Comm., 1997, 23 9:197  Pauli et al. J Allergy Clin Immunol, 1996, 97:1100-9 van Neerven et al. Clin Exp Allergy, 1998, 28:423-33 Jahn-Schmid et al. Immunotechnology, 1996, 2:103-13 Breitwieser et al. Biotechniques, 1996, 21:918-25

		Fuchs et al. J Allergy Clin Immunol, 1997, 100:3 56-64
Brazil nut	globulin	Bartolome et al. Allergol Immunopathol, 1997,25:135-44
Cherry	Pru a 1 (major allergen)	Scheurer et al. Mol Immunol, 1997, 34:619-29
Com	Zml3 (pollen)	Heiss et al. FEBS Lett, 1996, 381:217-21 Lehrer et al. Int Arch Allergy Immunol, 1997, 113:122-4
Grass	Phl p 1, Phl p 2, Phl p 5 (timothy grass pollen)	Bufe et al. Am J Respir Crit Care Med, 1998, 157:1269-76 Vrtala et al. J Immunol Jun 15, 1998, 160:6137-44 Niederberger et al. J Allergy Clin Immun., 1998, 101:258-64
	Hol 1 5 velvet grass pollen	Schramm et al. Eur J Biochem, 1998, 252:200-6
	Bluegrass allergen	Zhang et al. J Immunol, 1993, 151:791-9
	Cyn d 7 Bermuda grass	Smith et al. Int Arch Allergy Immunol, 1997, 114:265-71
	Cyn d 12 (a profilin)	Asturias et al. Clin Exp Allergy, 1997, 27:1307-13 Fuchs et al. J Allergy Clin Immunol, 1997, 100:356-64
Japanese Cedar	Jun a 2 (Juniperus ashei)	Yokoyama et al. Biochem. Biophys. Res. Commun., 2000, 275:195-202
	Cry j 1, Cry j 2 (Cryptomeria japonica)	Kingetsu et al. Immunology, 2000, 99:625-629
Juniper	Jun o 2 (pollen)	Tinghino et al. J Allergy Clin Immunol, 1998, 101:772-7
Latex	Hev b 7	Sowka et al. Eur J Biochem, 1998, 255:213-9 Fuchs et al. J Allergy Clin Immunol, 1997, 100:3 56-64
Mercurialis	Mer a 1 (profilin)	Vallverdu et al. J Allergy Clin Immunol, 1998, 101:3 63-70
Mustard (Yellow)	Sin a 1 (seed)	Gonzalez de la Pena et al. Biochem Bioph. Res Comm., 1993, 190:648-53
Oilseed rape	Bra r 1 pollen allergen	Smith et al. Int Arch Allergy Immunol, 1997, 114:265-71
Peanut	Ara h 1	Stanley et al. Adv Exp Med Biol, 1996, 409:213-6 Burks et al. J Clin Invest, 1995, 96:1715-21 Burks et al. Int Arch Allergy Immunol, 1995, 107:248-50
Poa pratensis	Poa p9	Parronchi et al. Eur J Immunol, 1996, 26:697-703 Astwood et al. Adv Exp Med Biol, 1996, 409:269-77
Ragweed	Amb a 1	Sun et al. Biotechnology Aug, 1995, 13:779-86 Hirschwehr et al. J Allergy Clin Immunol, 1998, 101:196-206 Casale et al. J Allergy Clin Immunol, 1997, 100:110-21
Rye	Lol p 1	Tamborini et al. Eur J Biochem, 1997, 249:886-94
Walnut	Jug r 1	Teuber et al. J Allergy Clin Immun., 1998, 101:807-14
Wheat	allergen	Fuchs et al. J Allergy Clin Immunol, 1997, 100:356-64 Donovan et al. Electrophoresis, 1993, 14:917-22
FUNGI:		
Aspergillus	Asp f 1, Asp f 2, Asp f3, Asp f 4, rAsp f 6	Crameri et al. Mycoses, 1998, 41 Suppl 1:56-60 Hemmann et al. Eur J Immunol, 1998, 28:1155-60 Banerjee et al. J Allergy Clin Immunol, 1997, 99:821-7 Crameri Int Arch Allergy Immunol, 1998, 115:99-114 Crameri et al. Adv Exp Med Biol, 1996, 409:111-6 Moser et al. J Allergy Clin Immunol, 1994, 93: 1-11
	Manganese superoxide	Mayer et al. Int Arch Allergy Immunol, 1997, 113:213-5

	dismutase (MNSOD)	
Blomia	allergen	Caraballo et al. Adv Exp Med Biol, 1996, 409:81-3
Penicillium	allergen	Shen et al. Clin Exp Allergy, 1997, 27:682-90
Psilocybe	Psi c 2	Horner et al. Int Arch Allergy Immunol, 1995, 107:298-300

**[0265]** In some embodiments, the antigen is from an infectious agent, including protozoan, bacterial, fungal (including unicellular and multicellular), and viral infectious agents. Examples of suitable viral antigens are described herein and are known in the art. Bacteria include *Hemophilus influenza*, *Mycobacterium tuberculosis* and *Bordetella pertussis*. Protozoan infectious agents include malarial plasmodia, *Leishmania* species, *Trypanosoma* species and *Schistosoma* species. Fungi include *Candida albicans*.

**[0266]** In some embodiments, the antigen is a viral antigen. Viral polypeptide antigens include, but are not limited to, HIV proteins such as HIV gag proteins (including, but not limited to, membrane anchoring (MA) protein, core capsid (CA) protein and nucleocapsid (NC) protein), HIV polymerase, influenza virus matrix (M) protein and influenza virus nucleocapsid (NP) protein, hepatitis B surface antigen (HBsAg), hepatitis B core protein (HBcAg), hepatitis e protein (HBeAg), hepatitis B DNA polymerase, hepatitis C antigens, and the like. References discussing influenza vaccination include Scherle and Gerhard (1988) *Proc. Natl. Acad. Sci. USA* 85:4446-4450; Scherle and Gerhard (1986) *J. Exp. Med.* 164:1114-1128; Granoff et al. (1993) *Vaccine* 11:S46-51; Kodihalli et al. (1997) *J. Virol.* 71:3391-3396; Ahmeida et al. (1993) *Vaccine* 11:1302-1309; Chen et al. (1999) *Vaccine* 17:653-659; Govorkova and Smirnov (1997) *Acta Virol.* (1997) 41:251-257; Koide et al. (1995) *Vaccine* 13:3-5; Mbawuike et al. (1994) *Vaccine* 12:1340-1348; Tamura et al. (1994) *Vaccine* 12:310-316; Tamura et al. (1992) *Eur. J. Immunol.* 22:477-481; Hirabayashi et al. (1990) *Vaccine* 8:595-599. Other examples of antigen polypeptides are group- or sub-group specific antigens, which are known for a number of infectious agents, including, but not limited to, adenovirus, herpes simplex virus, papilloma virus, respiratory syncytial virus and poxviruses.

**[0267]** Many antigenic peptides and proteins are known, and available in the art; others can be identified using conventional techniques. For immunization against tumor formation or treatment of existing tumors, immunomodulatory peptides can include tumor cells (live or irradiated), tumor cell extracts, or protein subunits of tumor antigens such as Her-2/neu, Mart1, carcinoembryonic antigen (CEA), gangliosides, human milk fat globule (HMFG), mucin (MUC1), MAGE antigens, BAGE antigens, GAGE antigens, gp100, prostate specific antigen (PSA), and tyrosinase. Vaccines for immuno-based contraception can be formed by including sperm proteins administered with CICs. Lea et al. (1996) *Biochim. Biophys. Acta* 1307:263.

**[0268]** Attenuated and inactivated viruses are suitable for use herein as the antigen. Preparation of these viruses is well-known in the art and many are commercially available (see, e.g., Physicians' Desk Reference (1998) 52nd edition, Medical Economics Company, Inc.). For example, polio virus is available as IPOL® (Pasteur Merieux Connaught) and ORIMUNE® (Lederle Laboratories), hepatitis A virus as VAQTA® (Merck), measles virus as ATTENUVAX® (Merck), mumps virus as MUMPSVAX® (Merck) and rubella virus as MERUVAX®II (Merck). Additionally, attenuated and inactivated viruses such as HIV-1, HIV-2, herpes simplex virus, hepatitis B virus, rotavirus, human and non-human papillomavirus and slow brain viruses can provide peptide antigens.

**[0269]** In some embodiments, the antigen comprises a viral vector, such as vaccinia, adenovirus, and canary pox.

**[0270]** Antigens may be isolated from their source using purification techniques known in the art or, more conveniently, may be produced using recombinant methods.

**[0271]** Antigenic peptides can include purified native peptides, synthetic peptides, recombinant proteins, crude protein extracts, attenuated or inactivated viruses, cells, micro-organisms, or fragments of such peptides. Immunomodulatory

peptides can be native or synthesized chemically or enzymatically. Any method of chemical synthesis known in the art is suitable. Solution phase peptide synthesis can be used to construct peptides of moderate size or, for the chemical construction of peptides, solid phase synthesis can be employed. Atherton et al. (1981) *Hoppe Seylers Z. Physiol. Chem.* 362:833-839. Proteolytic enzymes can also be utilized to couple amino acids to produce peptides. Kullmann (1987) *Enzymatic Peptide Synthesis*, CRC Press, Inc. Alternatively, the peptide can be obtained by using the biochemical machinery of a cell, or by isolation from a biological source. Recombinant DNA techniques can be employed for the production of peptides. Hames et al. (1987) *Transcription and Translation: A Practical Approach*, IRL Press. Peptides can also be isolated using standard techniques such as affinity chromatography.

[0272] Preferably the antigens are peptides, lipids (e.g., sterols excluding cholesterol, fatty acids, and phospholipids), polysaccharides such as those used in H. influenza vaccines, gangliosides and glycoproteins. These can be obtained through several methods known in the art, including isolation and synthesis using chemical and enzymatic methods. In certain cases, such as for many sterols, fatty acids and phospholipids, the antigenic portions of the molecules are commercially available.

[0273] Examples of viral antigens useful in the subject compositions and methods using the compositions include, but are not limited to, HIV antigens. Such antigens include, but are not limited to, those antigens derived from HIV envelope glycoproteins including, but not limited to, gp160, gp120 and gp41. Numerous sequences for HIV genes and antigens are known. For example, the Los Alamos National Laboratory HIV Sequence Database collects, curates and annotates HIV nucleotide and amino acid sequences. This database is accessible via the internet, at <http://hiv-web.lanl.gov/>, and in a yearly publication, see *Human Retroviruses and AIDS Compendium* (for example, 2000 edition).



[0274] Antigens derived from infectious agents may be obtained using methods known in the art, for example, from native viral or bacterial extracts, from cells infected with the infectious agent, from purified polypeptides, from recombinantly produced polypeptides and/or as synthetic peptides.

[0275] CICs can be administered in combination with antigen in a variety of ways. In some embodiments, a CIC and antigen are administered spatially proximate with respect to each other. As described below, spatial proximation can be accomplished in a number of ways, including conjugation, encapsidation, via affixation to a platform or adsorption onto a surface. In one embodiment, a CIC and antigen are administered as an admixture (*e.g.*, in solution). It is specifically contemplated that, in certain embodiments, the CIC is not conjugated to an immunogen or antigen.

[0276] In some embodiments, the CIC is linked to a polypeptide, *e.g.*, an antigen. The CIC portion can be linked with the antigen portion of a conjugate in a variety of ways, including covalent and/or non-covalent interactions, via the nucleic acid moiety or non-nucleic acid spacer moiety. In some embodiments, linkage is via a reactive group such as, without limitation, thio, amine, carboxylate, aldehyde, hydrazine, hydrazide, disulfide and the like.

[0277] The link between the portions can be made at the 3' or 5' end of a nucleic acid moiety, or at a suitably modified base at an internal position in the a nucleic acid moiety. For example, if the antigen is a peptide and contains a suitable reactive group (*e.g.*, an N-hydroxysuccinimide ester) it can be reacted directly with the N<sup>4</sup> amino group of cytosine residues. Depending on the number and location of cytosine residues in the CIC, specific coupling at one or more residues can be achieved.

[0278] Alternatively, modified oligonucleosides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the CIC. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, the antigen of interest.

[0279] Where the antigen is a peptide, this portion of the conjugate can be attached to the nucleic acid moiety or spacer moiety through solid support chemistry. For example, a nucleic acid portion of a CIC can be added to a polypeptide portion that has been pre-synthesized on a support. Haralambidis et al. (1990) *Nucleic Acids Res.* 18:493-499; and Haralambidis et al. (1990) *Nucleic Acids Res.* 18:501-505.

[0280] Alternatively, the CIC can be synthesized such that it is connected to a solid support through a cleavable linker extending from the 3'-end of a nucleic acid moiety. Upon chemical cleavage of the CIC from the support, a terminal thiol group or a terminal amino group is left at the 3'-end of the nucleic acid moiety (Zuckermann et al., 1987, *Nucleic Acids Res.* 15:5305-5321; Corey et al., 1987, *Science* 238:1401-1403; Nelson et al., 1989, *Nucleic Acids Res.* 17:1781-1794). Conjugation of the amino-modified CIC to amino groups of the peptide can be performed as described in Benoit et al. (1987) *Neuromethods* 6:43-72. Conjugation of the thiol-modified CIC to carboxyl groups of the peptide can be performed as described in Sinah et al. (1991) *Oligonucleotide Analogues: A Practical Approach*, IRL Press. Coupling of a nucleic acid moiety or spacer carrying an appended maleimide to the thiol side chain of a cysteine residue of a peptide has also been described. Tung et al. (1991) *Bioconjug. Chem.* 2:464-465.

[0281] The peptide portion of the conjugate can be attached to a free 5'-end of a nucleic acid moiety through an amine, thiol, or carboxyl group that has been incorporated into nucleic acid moiety or spacer (e.g., via a free 5'-end, a 3'-end, via a modified base, and the like).

[0282] Conveniently, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite can be covalently attached to a hydroxyl group of a CIC. Agrawal et al. (1986) *Nucleic Acids Res.* 14:6227-6245; Connolly (1985) *Nucleic Acids Res.* 13:4485-4502; Kremsky et al. (1987) *Nucleic Acids Res.* 15:2891-2909; Connolly (1987) *Nucleic Acids Res.* 15:3131-3139; Bischoff et al. (1987) *Anal. Biochem.* 164:336-344; Blanks et al. (1988) *Nucleic Acids Res.*

16:10283-10299; and U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802. Subsequent to deprotection, the amine, thiol, and carboxyl functionalities can be used to covalently attach the CIC to a peptide. Benoit et al. (1987); and Sinah et al. (1991).

[0283] A CIC-antigen conjugate can also be formed through non-covalent interactions, such as ionic bonds, hydrophobic interactions, hydrogen bonds and/or van der Waals attractions.

[0284] Non-covalently linked conjugates can include a non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of a CIC. Roget et al. (1989) *Nucleic Acids Res.* 17:7643-7651. Incorporation of a streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated oligonucleotide.

[0285] Non-covalent associations can also occur through ionic interactions involving a CIC and residues within the antigen, such as charged amino acids, or through the use of a linker portion comprising charged residues that can interact with both the oligonucleotide and the antigen. For example, non-covalent conjugation can occur between a generally negatively-charged CIC and positively-charged amino acid residues of a peptide, e.g., polylysine, polyarginine and polyhistidine residues.

[0286] Non-covalent conjugation between CIC and antigens can occur through DNA binding motifs of molecules that interact with DNA as their natural ligands. For example, such DNA binding motifs can be found in transcription factors and anti-DNA antibodies.

[0287] The linkage of the CIC to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugates (Yanagawa et al. (1988) *Nucleic Acids Symp. Ser.* 19:189-192), oligonucleotide-fatty acid conjugates (Grabarek et al. (1990) *Anal. Biochem.* 185:131-135; and Staros et al. (1986) *Anal. Biochem.* 156:220-222), and

oligonucleotide-sterol conjugates. Boujrad et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5728-5731.

[0288] The linkage of the oligonucleotide to an oligosaccharide can be formed using standard known methods. These methods include, but are not limited to, the synthesis of oligonucleotide-oligosaccharide conjugates, wherein the oligosaccharide is a moiety of an immunoglobulin. O'Shannessy et al. (1985) *J. Applied Biochem.* 7:347-355.

[0289] Additional methods for the attachment of peptides and other molecules to oligonucleotides can be found in U.S. Patent No. 5,391,723; Kessler (1992) "Nonradioactive labeling methods for nucleic acids" in Kricka (ed.) *Nonisotopic DNA Probe Techniques*, Academic Press; and Geoghegan et al. (1992) *Bioconjug. Chem.* 3:138-146.

[0290] A CIC may be proximately associated with an antigen(s) in other ways. In some embodiments, a CIC and antigen are proximately associated by encapsulation. In other embodiments, a CIC and antigen are proximately associated by linkage to a platform molecule. A "platform molecule" (also termed "platform") is a molecule containing sites which allow for attachment of the a CIC and antigen(s). In other embodiments, a CIC and antigen are proximately associated by adsorption onto a surface, preferably a carrier particle.

[0291] In some embodiments, the methods of the invention employ an encapsulating agent that can maintain the proximate association of the a CIC and first antigen until the complex is available to the target (or compositions comprising such encapsulating agents). Preferably, the composition comprising a CIC, antigen and encapsulating agent is in the form of adjuvant oil-in-water emulsions, microparticles and/or liposomes. More preferably, adjuvant oil-in-water emulsions, microparticles and/or liposomes encapsulating a CIC are in the form of particles from about 0.04  $\mu\text{m}$  to about 100  $\mu\text{m}$  in size, preferably any of the following ranges: from about 0.1  $\mu\text{m}$

to about 20  $\mu\text{m}$ ; from about 0.15  $\mu\text{m}$  to about 10  $\mu\text{m}$ ; from about 0.05  $\mu\text{m}$  to about 1.00  $\mu\text{m}$ ; from about 0.05  $\mu\text{m}$  to about 0.5  $\mu\text{m}$ .

**[0292]** Colloidal dispersion systems, such as microspheres, beads, macromolecular complexes, nanocapsules and lipid-based systems, such as oil-in-water emulsions, micelles, mixed micelles and liposomes can provide effective encapsulation of CIC-containing compositions.

**[0293]** The encapsulation composition further comprises any of a wide variety of components. These include, but are not limited to, alum, lipids, phospholipids, lipid membrane structures (LMS), polyethylene glycol (PEG) and other polymers, such as polypeptides, glycopeptides, and polysaccharides.

**[0294]** Polypeptides suitable for encapsulation components include any known in the art and include, but are not limited to, fatty acid binding proteins. Modified polypeptides contain any of a variety of modifications, including, but not limited to glycosylation, phosphorylation, myristylation, sulfation and hydroxylation. As used herein, a suitable polypeptide is one that will protect a CIC-containing composition to preserve the immunomodulatory activity thereof. Examples of binding proteins include, but are not limited to, albumins such as bovine serum albumin (BSA) and pea albumin.

**[0295]** Other suitable polymers can be any known in the art of pharmaceuticals and include, but are not limited to, naturally-occurring polymers such as dextrans, hydroxyethyl starch, and polysaccharides, and synthetic polymers. Examples of naturally occurring polymers include proteins, glycopeptides, polysaccharides, dextran and lipids. The additional polymer can be a synthetic polymer. Examples of synthetic polymers which are suitable for use in the present invention include, but are not limited to, polyalkyl glycols (PAG) such as PEG, polyoxyethylated polyols (POP), such as polyoxyethylated glycerol (POG), polytrimethylene glycol (PTG) polypropylene glycol (PPG), polyhydroxyethyl methacrylate, polyvinyl alcohol (PVA), polyacrylic acid, polyethyloxazoline, polyacrylamide, polyvinylpyrrolidone

(PVP), polyamino acids, polyurethane and polyphosphazene. The synthetic polymers can also be linear or branched, substituted or unsubstituted, homopolymeric, co-polymers, or block co-polymers of two or more different synthetic monomers.

[0296] The PEGs for use in encapsulation compositions of the present invention are either purchased from chemical suppliers or synthesized using techniques known to those of skill in the art.

[0297] The term "LMS", as used herein, means lamellar lipid particles wherein polar head groups of a polar lipid are arranged to face an aqueous phase of an interface to form membrane structures. Examples of the LMSs include liposomes, micelles, cochleates (*i.e.*, generally cylindrical liposomes), microemulsions, unilamellar vesicles, multilamellar vesicles, and the like.

[0298] One colloidal dispersion system useful in the administration of CICs is a liposome. In mice immunized with a liposome-encapsulated antigen, liposomes appeared to enhance a Th1-type immune response to the antigen. Aramaki et al. (1995) *Vaccine* 13:1809-1814. As used herein, a "liposome" or "lipid vesicle" is a small vesicle bounded by at least one and possibly more than one bilayer lipid membrane. Liposomes are made artificially from phospholipids, glycolipids, lipids, steroids such as cholesterol, related molecules, or a combination thereof by any technique known in the art, including but not limited to sonication, extrusion, or removal of detergent from lipid-detergent complexes. One type of liposome for use in delivering CICs to cells is a cationic liposome. A liposome can also optionally comprise additional components, such as a tissue targeting component. It is understood that a "lipid membrane" or "lipid bilayer" need not consist exclusively of lipids, but can additionally contain any suitable other components, including, but not limited to, cholesterol and other steroids, lipid-soluble chemicals, proteins of any length, and other amphipathic molecules, providing the general structure of the membrane is a sheet of two hydrophilic surfaces sandwiching a hydrophobic core. For a general discussion of membrane structure, see *The Encyclopedia of Molecular*

*Biology* by J. Kendrew (1994). For suitable lipids see *e.g.*, Lasic (1993) "Liposomes: from Physics to Applications" Elsevier, Amsterdam.

[0299] Processes for preparing liposomes containing CIC-containing compositions are known in the art. The lipid vesicles can be prepared by any suitable technique known in the art. Methods include, but are not limited to, microencapsulation, microfluidization, LLC method, ethanol injection, freon injection, the "bubble" method, detergent dialysis, hydration, sonication, and reverse-phase evaporation. Reviewed in Watwe et al. (1995) *Curr. Sci.* 68:715-724. Techniques may be combined in order to provide vesicles with the most desirable attributes.

[0300] The invention encompasses use of LMSs containing tissue or cellular targeting components. Such targeting components are components of a LMS that enhance its accumulation at certain tissue or cellular sites in preference to other tissue or cellular sites when administered to an intact animal, organ, or cell culture. A targeting component is generally accessible from outside the liposome, and is therefore preferably either bound to the outer surface or inserted into the outer lipid bilayer. A targeting component can be *inter alia* a peptide, a region of a larger peptide, an antibody specific for a cell surface molecule or marker, or antigen binding fragment thereof, a nucleic acid, a carbohydrate, a region of a complex carbohydrate, a special lipid, or a small molecule such as a drug, hormone, or hapten, attached to any of the aforementioned molecules. Antibodies with specificity toward cell type-specific cell surface markers are known in the art and are readily prepared by methods known in the art.

[0301] The LMSs can be targeted to any cell type toward which a therapeutic treatment is to be directed, *e.g.*, a cell type which can modulate and/or participate in an immune response. Such target cells and organs include, but are not limited to, APCs, such as macrophages, dendritic cells and lymphocytes, lymphatic structures,

such as lymph nodes and the spleen, and nonlymphatic structures, particularly those in which dendritic cells are found.

**[0302]** The LMS compositions of the present invention can additionally comprise surfactants. Surfactants can be cationic, anionic, amphiphilic, or nonionic. A preferred class of surfactants are nonionic surfactants; particularly preferred are those that are water soluble.

**[0303]** In some embodiments a CIC and antigen are proximately associated by linkage to a platform molecule, such as a proteinaceous or non-proteinaceous (*e.g.*, synthetic) valency platform. Examples of suitable platforms are described *supra*, in the discussion of valency platforms used as a spacer moiety in a CIC. Attachment of antigens to valency platforms can be carried out using routine methods. As an example, polypeptides contain amino acid side chain moieties with functional groups such as amino, carboxyl or sulfhydryl groups that serve as sites for coupling the polypeptide to the platform. Residues that have such functional groups may be added to the polypeptide if the polypeptide does not already contain these groups. Such residues may be incorporated by solid phase synthesis techniques or recombinant techniques, both of which are well known in the peptide synthesis arts. When the polypeptide has a carbohydrate side chain(s) (or if the antigen is a carbohydrate), functional amino, sulfhydryl and/or aldehyde groups may be incorporated therein by conventional chemistry. For instance, primary amino groups may be incorporated by reaction of the oxidized sugar with ethylenediamine in the presence of sodium cyanoborohydride, sulfhydryls may be introduced by reaction of cysteamine dihydrochloride followed by reduction with a standard disulfide reducing agent, while aldehyde groups may be generated following periodate oxidation. In a similar fashion, the platform molecule may also be derivatized to contain functional groups if it does not already possess appropriate functional groups.

**[0304]** In another embodiment, a CIC and antigen are coadministered by adsorbing both to a surface, such as a nanoparticle or microcarrier. Adsorption of a



CIC and/or antigen to a surface may occur through non-covalent interactions, including ionic and/or hydrophobic interactions. Adsorption of polynucleotides and polypeptides to a surface for the purpose of delivery of the adsorbed molecules to cells is well known in the art. See, for example, Douglas et al. (1987) *Crit. Rev. Ther. Drug. Carrier Syst.* 3:233-261; Hagiwara et al. (1987) *In Vivo* 1:241-252; Bousquet et al. (1999) *Pharm. Res.* 16:141-147; and Kossovsky et al., U.S. Patent 5,460,831. Preferably, the material comprising the adsorbent surface is biodegradable.

**[0305]** In general, characteristics of nanoparticles, such as surface charge, particle size and molecular weight, depend upon polymerization conditions, monomer concentration and the presence of stabilizers during the polymerization process (Douglas et al., 1987, *supra*). The surface of carrier particles may be modified, for example, with a surface coating, to allow or enhance adsorption of the CIC and/or antigen. Carrier particles with adsorbed CIC and/or antigen may be further coated with other substances. The addition of such other substances may, for example, prolong the half-life of the particles once administered to the subject and/or may target the particles to a specific cell type or tissue, as described herein.

**[0306]** Nanocrystalline surfaces to which a CIC and antigen may be adsorbed have been described (see, for example, U.S. Patent 5,460,831). Nanocrystalline core particles (with diameters of 1  $\mu\text{m}$  or less) are coated with a surface energy modifying layer that promotes adsorption of polypeptides, polynucleotides and/or other pharmaceutical agents. As described in U.S. Patent 5,460,831, for example, a core particle is coated with a surface that promotes adsorption of an oligonucleotide and is subsequently coated with an antigen preparation, for example, in the form of a lipid-antigen mixture. Such nanoparticles are self-assembling complexes of nanometer sized particles, typically on the order of 0.1  $\mu\text{m}$ , that carry an inner layer of CIC and an outer layer of antigen.

**[0307]** Another adsorbent surface are nanoparticles made by the polymerization of alkylcyanoacrylates. Alkylcyanoacrylates can be polymerized in acidified aqueous

media by a process of anionic polymerization. Depending on the polymerization conditions, the small particles tend to have sizes in the range of 20 to 3000 nm, and it is possible to make nanoparticles specific surface characteristics and with specific surface charges (Douglas et al., 1987, *supra*). For example, oligonucleotides may be adsorbed to polyisobutyl- and polyisohexylcyanoacrylate nanoparticles in the presence of hydrophobic cations such as tetraphenylphosphonium chloride or quaternary ammonium salts, such as cetyltrimethyl ammonium bromide. Oligonucleotide adsorption on these nanoparticles appears to be mediated by the formation of ion pairs between negatively charged phosphate groups of the nucleic acid chain and the hydrophobic cations. See, for example, Lambert et al. (1998) *Biochimie* 80:969-976, Chavany et al. (1994) *Pharm. Res.* 11:1370-1378; Chavany et al. (1992) *Pharm. Res.* 9:441-449. Polypeptides may also be adsorbed to polyalkylcyanoacrylate nanoparticles. See, for example, Douglas et al., 1987; Schroeder et al. (1998) *Peptides* 19:777-780.

[0308] Another adsorbent surface are nanoparticles made by the polymerization of methyldene malonate. For example, as described in Bousquet et al., 1999, polypeptides adsorbed to poly(methyldene malonate 2.1.2) nanoparticles appear to do so initially through electrostatic forces followed by stabilization through hydrophobic forces.

### C. Additional Adjuvants

[0309] A CIC may also be administered in conjunction with an adjuvant. Administration of an antigen with a CIC and an adjuvant leads to a potentiation of a immune response to the antigen and thus, can result in an enhanced immune response compared to that which results from a composition comprising the CIC and antigen alone. Adjuvants are known in the art and include, but are not limited to, oil-in-water emulsions, water-in oil emulsions, alum (aluminum salts), liposomes and microparticles, including but not limited to, polystyrene, starch, polyphosphazene and

polylactide/polyglycosides. Other suitable adjuvants also include, but are not limited to, MF59, DETOX™ (Ribi), squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) *Nature* 344:873-875, as well as, lipid-based adjuvants and others described herein. For veterinary use and for production of antibodies in animals, mitogenic components of Freund's adjuvant (both complete and incomplete) can be used.

#### IV. Methods of the Invention

**[0310]** The invention provides methods of modulating an immune response of an animal or population of cells, e.g., mammalian, optionally human, blood cells (e.g., PBMCs, lymphocytes, dendritic cells), bronchial alveolar lavage cells, or other cells or cell populations containing cells responsive to immunostimulatory agents, by contacting the cells with a CIC or CIC-containing composition described herein (e.g., a composition containing a CIC, CIC and an antigen, a CIC-antigen conjugate, a CIC/microcarrier complex, etc.) The modulation can be accomplished by any form of contacting, including without limitation, co-incubation of cells and CIC *in vitro*, application of the CIC to skin of a mammal (e.g., of an experimental animal), and parenteral administration.

**[0311]** An immune response in animals or cell populations can be detected in any number of ways, including a increased expression of one or more of IFN- $\gamma$ , IFN- $\alpha$ , IL-2, IL-12, TNF- $\alpha$ , IL-6, IL-4, IL-5, IP-10, ISG-54K, MCP-1, or a change in gene expression profile characteristic of immune stimulation (see, e.g., Example 43) as well as responses such as B cell proliferation and dendritic cell maturation. The ability to stimulate an immune response in a cell population has a number of uses, e.g., in an assay system for immunosuppressive agents.

[0312] Thus, the invention provides methods of modulating an immune response in an individual, preferably a mammal, more preferably a human, comprising administering to the individual a CIC as described herein. Immunomodulation may include stimulating a Th1-type immune response and/or inhibiting or reducing a Th2-type immune response. The CIC is administered in an amount sufficient to modulate an immune response. As described herein, modulation of an immune response may be humoral and/or cellular, and is measured using standard techniques in the art and as described herein.

[0313] In certain embodiments, the individual suffers from a disorder associated with a Th2-type immune response, such as (without limitation) allergies, allergy-induced asthma, atopic dermatitis, eosinophilic gastrointestinal inflammation, eosinophilic esophagitis, and allergic bronchopulmonary aspergillosis.

Administration of a CIC results in immunomodulation, increasing levels of one or more Th1-type response associated cytokines, which may result in a reduction of the Th2-type response features associated with the individual's response to the allergen.

Immunomodulation of individuals with Th2-type response associated disorders results in a reduction or improvement in one or more of the symptoms of the disorder.

Where the disorder is allergy or allergy-induced asthma, improvement in one or more of the symptoms includes a reduction one or more of the following: rhinitis, allergic conjunctivitis, circulating levels of IgE, circulating levels of histamine and/or requirement for 'rescue' inhaler therapy (e.g., inhaled albuterol administered by metered dose inhaler or nebulizer).

[0314] In further embodiments, the individual subject to the immunomodulatory therapy of the invention is an individual receiving a vaccine. The vaccine may be a prophylactic vaccine or a therapeutic vaccine. A prophylactic vaccine comprises one or more epitopes associated with a disorder for which the individual may be at risk (e.g., *M. tuberculosis* antigens as a vaccine for prevention of tuberculosis).

Therapeutic vaccines comprise one or more epitopes associated with a particular

disorder affecting the individual, such as *M. tuberculosis* or *M. bovis* surface antigens in tuberculosis patients, antigens to which the individual is allergic (*i.e.*, allergy desensitization therapy) in individuals subject to allergies, tumor cells from an individual with cancer (*e.g.*, as described in U.S. Patent No. 5,484,596), or tumor associated antigens in cancer patients. The CIC may be given in conjunction with the vaccine (*e.g.*, in the same injection or a contemporaneous, but separate, injection) or the CIC may be administered separately (*e.g.*, at least 12 hours before or after administration of the vaccine). In certain embodiments, the antigen(s) of the vaccine is part of the CIC, by either covalent or non-covalent linkage to the CIC.

Administration of CIC therapy to an individual receiving a vaccine results in an immune response to the vaccine that is shifted towards a Th1-type response as compared to individuals which receive vaccine not containing a CIC. Shifting towards a Th1-type response may be recognized by a delayed-type hypersensitivity (DTH) response to the antigen(s) in the vaccine, increased IFN- $\gamma$  and other Th1-type response associated cytokines, production of CTLs specific for the antigen(s) of the vaccine, low or reduced levels of IgE specific for the antigen(s) of the vaccine, a reduction in Th2-associated antibodies specific for the antigen(s) of the vaccine, and/or an increase in Th1-associated antibodies specific for the antigen(s) of the vaccine. In the case of therapeutic vaccines, administration of CIC and vaccine also results in amelioration of one or more symptoms of the disorder which the vaccine is intended to treat. As will be apparent to one of skill in the art, the exact symptoms and manner of their improvement will depend on the disorder sought to be treated. For example, where the therapeutic vaccine is for tuberculosis, CIC treatment with vaccine results in reduced coughing, pleural or chest wall pain, fever, and/or other symptoms known in the art. Where the vaccine is an allergen used in allergy desensitization therapy, the treatment results in a reduction in the symptoms of allergy (*e.g.*, reduction in rhinitis, allergic conjunctivitis, circulating levels of IgE, and/or circulating levels of histamine).

[0315] The compositions of the invention may also be used prophylactically to increase resistance to infection by a wide range of bacterial or viral pathogens, including natural or genetically modified organisms employed as agents of biological warfare or terrorism.

[0316] Other embodiments of the invention relate to immunomodulatory therapy of individuals having a pre-existing disease or disorder, such as cancer or an infectious disease. Cancer is an attractive target for immunomodulation because most cancers express tumor-associated and/or tumor specific antigens which are not found on other cells in the body. Stimulation of a Th1-type response against tumor cells results in direct and/or bystander killing of tumor cells by the immune system, leading to a reduction in cancer cells and a reduction in symptoms. Administration of a CIC to an individual having cancer results in stimulation of a Th1-type immune response against the tumor cells. Such an immune response can kill tumor cells, either by direct action of cellular immune system cells (*e.g.*, CTLs) or components of the humoral immune system, or by bystander effects on cells proximal to cells targeted by the immune system including macrophages and natural killer (NK) cells. See, for example, Cho et al. (2000) *Nat. Biotechnol.* 18:509-514. In treatment of a pre-existing disease or disorder, the CIC can be administered in conjunction with other immunotherapeutic agents such as cytokines, adjuvants and antibodies. For example, a CIC can be administered as part of a therapeutic regimen that includes administration of a binding agent that binds an antigen displayed by tumor cells. Exemplary binding agents include polyclonal and monoclonal antibodies. Examples of target antigens include CD20, CD22, HER2 and others known in the art or to be discovered in the future. Without intending to be bound by theory, it is believed that the CIC enhances killing of tumor cells to which the binding agent is associated (*e.g.*, by enhancing antibody dependent cellular cytotoxicity and/or effector function). The binding agent can optionally be labeled, *e.g.*, with a radioisotope or toxin that damages a cell to which the binding agent is bound. The CIC may be given in

conjunction with the agent (e.g., at the same time) or before or after (e.g., less than 24 hours before or after administration of the agent). For example, in the case of cancer, the CIC can be administered in conjunction with a chemotherapeutic agent known or suspected of being effective for the treatment of cancer. As another example, the CIC can be administered in conjunction with radiation therapy, gene therapy, or the like. The CIC may be any of those described herein.

[0317] Immunomodulatory therapy in accordance with the invention is also beneficial for individuals with infectious diseases, particularly infectious diseases which are resistant to humoral immune responses (e.g., diseases caused by mycobacterial infections and intracellular pathogens). Immunomodulatory therapy may be used for the treatment of infectious diseases caused by cellular pathogens (e.g., bacteria or protozoans) or by subcellular pathogens (e.g., viruses). CIC therapy may be administered to individuals suffering from mycobacterial diseases such as tuberculosis (e.g., *M. tuberculosis* and/or *M. bovis* infections), leprosy (i.e., *M. leprae* infections), or *M. marinum* or *M. ulcerans* infections. CIC therapy is also may also be used for the treatment of viral infections, including infections by influenza virus, respiratory syncytial virus (RSV), hepatitis virus B, hepatitis virus C, herpes viruses, particularly herpes simplex viruses, and papilloma viruses. Diseases caused by intracellular parasites such as malaria (e.g., infection by *Plasmodium vivax*, *P. ovale*, *P. falciparum* and/or *P. malariae*), leishmaniasis (e.g., infection by *Leishmania donovani*, *L. tropica*, *L. mexicana*, *L. braziliensis*, *L. peruviana*, *L. infantum*, *L. chagasi*, and/or *L. aethiopica*), and toxoplasmosis (i.e., infection by *Toxoplasmosis gondii*) also benefit from CIC therapy. CIC therapy may also be used for treatment of parasitic diseases such as schistosomiasis (i.e., infection by blood flukes of the genus *Schistosoma* such as *S. haematobium*, *S. mansoni*, *S. japonicum*, and *S. mekongi*) and clonorchiasis (i.e., infection by *Clonorchis sinensis*). Administration of a CIC to an individual suffering from an infectious disease results in an amelioration of

symptoms of the infectious disease. In some embodiments, the infectious disease is not a viral disease.

**[0318]** The invention further provides methods of increasing or stimulating at least one Th1-associated cytokine in an individual, including IL-2, IL-12, TNF- $\alpha$ , TNF- $\beta$ , IFN- $\gamma$  and IFN- $\alpha$ . In certain embodiments, the invention provides methods of increasing or stimulating IFN- $\gamma$  in an individual, particularly in an individual in need of increased IFN- $\gamma$  levels, by administering an effective amount of a CIC to the individual. Individuals in need of increased IFN- $\gamma$  are those having disorders which respond to the administration of IFN- $\gamma$ . Such disorders include a number of inflammatory disorders including, but not limited to, ulcerative colitis. Such disorders also include a number of fibrotic disorders, including, but not limited to, idiopathic pulmonary fibrosis (IPF), scleroderma, cutaneous radiation-induced fibrosis, hepatic fibrosis including schistosomiasis-induced hepatic fibrosis, renal fibrosis as well as other conditions which may be improved by administration of IFN- $\gamma$ . An increase in IFN- $\gamma$  levels may result in amelioration of one or more symptoms, stabilization of one or more symptoms, or prevention of progression (*e.g.*, reduction or elimination of additional lesions or symptoms) of the disorder which responds to IFN- $\gamma$ . The methods of the invention may be practiced in combination with other therapies which make up the standard of care for the disorder, such as administration of anti-inflammatory agents such as systemic corticosteroid therapy (*e.g.*, cortisone) in IPF.

**[0319]** In certain embodiments, the invention provides methods of increasing IFN- $\alpha$  in an individual, particularly in an individual in need of increased IFN- $\alpha$  levels, by administering an effective amount of a CIC to the individual such that IFN- $\alpha$  levels are increased. Individuals in need of increased IFN- $\alpha$  are those having disorders which respond to the administration of IFN- $\alpha$ , including recombinant IFN- $\alpha$ , including, but not limited to, viral infections and cancer.



[0320] Administration of a CIC in accordance with certain embodiments of the invention results in an increase in IFN- $\alpha$  levels, and results in amelioration of one or more symptoms, stabilization of one or more symptoms, or prevention of progression (e.g., reduction or elimination of additional lesions or symptoms) of the disorder which responds to IFN- $\alpha$ . The methods of the invention may be practiced in combination with other therapies which make up the standard of care for the disorder, such as administration of anti-viral agents for viral infections.

[0321] As will be apparent upon review of this disclosure, the spacer composition of a CIC can affect the immune response elicited by administration of the CIC. Virtually all of the spacers tested (with the exception of dodecyl) can be used in CICs to efficiently induce IFN- $\gamma$  in human PBMCs. However, the spacer composition of linear CICs has been observed to have differential effects on induction of IFN- $\alpha$ . For example, CICs containing, for example, HEG, TEG or C6 spacers tend to cause higher IFN- $\alpha$  induction (and reduced B cell proliferation) in PBMCs than did CICs containing C3, C4 or abasic spacers (see, e.g., Example 34, *infra*).

[0322] The invention also provides methods of reducing levels, particularly serum levels, of IgE in an individual having an IgE-related disorder by administering an effective amount of a CIC to the individual. In such methods, the CIC may be administered alone (e.g., without antigen) or administered with antigen, such as an allergen. An IgE-related disorder is a condition, disorder, or set of symptoms ameliorated by a reduction in IgE levels. Reduction in IgE results in an amelioration of symptoms of the IgE-related disorder. Such symptoms include allergy symptoms such as rhinitis, conjunctivitis, in decreased sensitivity to allergens, a reduction in the symptoms of allergy in an individual with allergies, or a reduction in severity of an allergic response.

[0323] Guided by the present disclosure, CICs can be designed to achieve specific desired physiological responses. For example, the IFN- $\alpha$  inducing activity, and B cell proliferation-inducing activities of CICs can be independently varied

based on the structure of the CIC and selection of NAMs. For example, as illustrated in Fig. 10, IFN- $\alpha$  production was effectively stimulated by CICs containing the sequences 5'-TCGXCGX and 5'-TCGXTCG (e.g., <sup>F</sup>5'-TCGXCGX and <sup>F</sup>5'-TCGXTCG) where X is any nucleotide. (It will be appreciated by the reader that other CIC structures can also effectively stimulate IFN- $\alpha$  production.) In addition, induction of IFN- $\alpha$  was significantly enhanced by multivalent CICs that present multiple copies NAMs of the aforementioned motifs linked to long, hydrophilic spacer moieties (e.g., hexaethylene glycol).

[0324] The effect of CIC structure (including nucleic acid moiety motifs and spacers) on B cell proliferation was also tested using purified peripheral blood B cells. Dose titration was performed in order to determine the optimal concentration for the assay, which was determined to be 5 mg/ml (data not shown). CICs containing heptameric motifs with a 5'-TCGT induced the highest levels of proliferation, while, surprisingly, many CICs containing 5'-TCGA sequences stimulated only low levels or background levels of proliferation. Comparing CICs containing identical branched CIC structures, spacers, and sequences, with the exception of the nucleotide following the 5'-TCG in each motif, confirmed that TCGT sequences were significantly more active than TCGA sequences, with TCGC and TCGG sequences having intermediate activity. The bases following the 5'-TCGX also had some influence on B cell activity. We found that C-74, containing the nucleic acid moiety motif TCGATTT, induced moderate levels of B cell proliferation, which were intermediate between the low to background levels found for other TCGA-containing CICs and the levels observed for C-41, containing the sequence TCGTTTT, and other TCGT-containing CICs. No significant differences in proliferation were observed for linear (C-21) vs. branched CICs (C-94) or for CICs containing different types of spacers (compare C-94, C-103, and C-104). From these data it appears that stimulation of B cell activity is largely a function of the sequence

of the nucleic acid moieties, with spacers and multimeric presentation being less significant.

**[0325]** The data presented in Figures 10 and 11 illustrate of the ability to independently vary B cell proliferation-inducing and IFN- $\alpha$ -inducing activity. For example, C-101, C-125, and C-145 all induce high levels of IFN- $\alpha$ , but induce very little B cell proliferation. C-94, C-142, and C-158 induce high levels of IFN- $\alpha$ , and also induce B cells to proliferate. Finally, C-104 induces no measurable IFN- $\alpha$ , but stimulates B cells to proliferate.

**[0326]** Because the IFN- $\alpha$  inducing activity and B cell proliferation-inducing activities of CICs can be independently varied based on the structure of the CIC and selection of NAMs it is possible to identify and produce CICs with different levels of each of these activities, using screening methods described herein and the information about B cell stimulating activity described herein. For example, CICs can be designed to exhibit different B cell proliferation-inducing activities, from insignificant up to levels equivalent to P-6, independently of the amount of IFN- $\alpha$  induced by that CIC. Similarly, CICs with different levels of IFN-inducing activity can be identified and produced.

**[0327]** Thus, without limitation, in one aspect, the invention provides CICs that induce IFN- $\alpha$  production and do not induce human B cell proliferation, and methods of using such CICs. In a related aspect, the invention provides CICs that induce IFN- $\alpha$  production and little human B cell proliferation and methods of using such CICs. In a related aspect, the invention provides design algorithms and screening methods for identifying CICs with these properties.

**[0328]** A CIC is considered to not induce human B cell proliferation if B cell proliferation in the presence of the CIC is at "background" levels, i.e., 0 to 15%, optionally 0 to about 10%, of the proliferation induced by an equal amount (e.g., 5  $\mu$ g/ml) of P-6. A CIC is considered to induce "little" human B cell proliferation if B cell proliferation in the presence of the CIC is between greater than 15 to about 30%

of P-6. Thus, in some embodiments a CIC of the invention induces less than about 30%, sometimes less than about 25%, sometimes less than about 20%, sometimes less than about 15% or less than about 10% of the level of B cell proliferation induced by P-6. For illustration and not limitation, examples of such CICs include: C-51; C-101; C-144; C-145; C-146; C-148; C-149; C-150. Alternatively, a CIC is considered to not induce human B cell proliferation if B cell proliferation in the presence of the CIC is not statistically significantly greater than the B cell proliferation induced by an equal concentration (e.g., 5 ug/ml) of a control chimeric compound, such as M-3, using an *in vitro* assay. See Fig. 11.

[0329] The ability to "program" CICs to exhibit different biological properties allows for the assembly of CICs exhibiting a defined set of activities tailored for specific clinical applications. For example, CICs with high IFN- $\alpha$  production and little B cell activation may be particularly useful in cancer therapies, while CICs with moderate IFN- $\alpha$  production and little B cell activation are particularly useful for treatment of diseases such as asthma. As previously noted, for certain indications, including the treatment of allergic asthma and certain cancers, it may be desirable to avoid polyclonal B cell activation, which might result in the potentiation of asthma-mediating B cells or B cell lymphomas. A variety of uses are known for CICs that preferentially stimulate B cell proliferation, including without limitation *in vivo* expansion to produce B cell clones for analysis.

[0330] Methods of the invention includes embodiments in which CICs are administered in the form of a CIC/microcarrier complex(s).

[0331] In some embodiments, the invention provides methods of stimulating CTL production in an individual, comprising administering an effective amount of a CIC to the individual such that CTL production is increased.

[0332] As will be apparent to one of skill in the art, the methods of the invention may be practiced in combination with other therapies for the particular indication for which the CIC is administered. For example, CIC therapy may be administered in

conjunction with anti-malarial drugs such as chloroquine for malaria patients, in conjunction with leishmanicidal drugs such as pentamidine and/or allopurinol for leishmaniasis patients, in conjunction with anti-mycobacterial drugs such as isoniazid, rifampin and/or ethambutol in tuberculosis patients, or in conjunction with allergen desensitization therapy for atopic (allergy) patients.

A. Administration and Assessment of the Immune Response

[0333] The CIC can be administered in combination with pharmaceutical and/or immunogenic and/or other immunostimulatory agents, as described herein, and can be combined with a physiologically acceptable carrier thereof.

[0334] For example, a CIC or composition of the invention can be administered in conjunction with other immunotherapeutic agents such as cytokines, adjuvants and antibodies. The CIC may be given in conjunction with the agent (*e.g.*, at the same time, or before or after (*e.g.*, less than 24 hours before or after administration of the agent)). The CIC may be any of those described herein.

[0335] As with all immunostimulatory compositions, the immunologically effective amounts and method of administration of the particular CIC formulation can vary based on the individual, what condition is to be treated and other factors evident to one skilled in the art. Factors to be considered include the presence of a coadministered antigen, whether or not the CIC will be administered with or covalently attached to an adjuvant or delivery molecule, route of administration and the number of immunizing doses to be administered. Such factors are known in the art and it is well within the skill of those in the art to make such determinations without undue experimentation. A suitable dosage range is one that provides the desired modulation of immune response to the antigen. Generally, dosage is determined by the amount of CIC administered to the patient, rather than the overall quantity of CIC. Exemplary dosage ranges of the CIC, given in amounts of CIC delivered, may be, for example, from about any of the following: 1 to 500  $\mu\text{g/kg}$ , 100

to 400 µg/kg, 200 to 300 µg/kg, 1 to 100 µg/kg, 100 to 200 µg/kg, 300 to 400 µg/kg, 400 to 500 µg/kg. The absolute amount given to each patient depends on pharmacological properties such as bioavailability, clearance rate and route of administration.

[0336] The effective amount and method of administration of the particular CIC formulation can vary based on the individual patient and the stage of the disease and other factors evident to one skilled in the art. The route(s) of administration suited for a particular application will be known to one of skill in the art. Routes of administration include but are not limited to topical, dermal, transdermal, transmucosal, epidermal, parenteral, gastrointestinal, and naso-pharyngeal and pulmonary, including transbronchial and transalveolar. A suitable dosage range is one that provides sufficient CIC-containing composition to attain a tissue concentration of about 1-10 µM as measured by blood levels. The absolute amount given to each patient depends on pharmacological properties such as bioavailability, clearance rate and route of administration.

[0337] As described herein, APCs and tissues with high concentration of APCs are preferred targets for the CIC. Thus, administration of CIC to mammalian skin and/or mucosa, where APCs are present in relatively high concentration, is preferred.

[0338] The present invention provides CIC formulations suitable for topical application including, but not limited to, physiologically acceptable implants, ointments, creams, rinses and gels. Topical administration is, for instance, by a dressing or bandage having dispersed therein a delivery system, by direct administration of a delivery system into incisions or open wounds, or by transdermal administration device directed at a site of interest. Creams, rinses, gels or ointments having dispersed therein a CIC are suitable for use as topical ointments or wound filling agents.

[0339] Preferred routes of dermal administration are those which are least invasive. Preferred among these means are transdermal transmission, epidermal

administration and subcutaneous injection. Of these means, epidermal administration is preferred for the greater concentrations of APCs expected to be in intradermal tissue.

**[0340]** Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the CIC to penetrate the skin and enter the blood stream. Compositions suitable for transdermal administration include, but are not limited to, pharmaceutically acceptable suspensions, oils, creams and ointments applied directly to the skin or incorporated into a protective carrier such as a transdermal device (so-called "patch"). Examples of suitable creams, ointments etc. can be found, for instance, in the Physician's Desk Reference.

**[0341]** For transdermal transmission, iontophoresis is a suitable method. Iontophoretic transmission can be accomplished using commercially available patches which deliver their product continuously through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

**[0342]** An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference. Other occlusive patch systems are also suitable.

**[0343]** For transdermal transmission, low-frequency ultrasonic delivery is also a suitable method. Mitragotri et al. (1995) *Science* 269:850-853. Application of low-frequency ultrasonic frequencies (about 1 MHz) allows the general controlled delivery of therapeutic compositions, including those of high molecular weight.

[0344] Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. Specifically, the irritation should be sufficient to attract APCs to the site of irritation.

[0345] An exemplary mechanical irritant means employs a multiplicity of very narrow diameter, short tines which can be used to irritate the skin and attract APCs to the site of irritation, to take up CIC transferred from the end of the tines. For example, the MONO-VACC old tuberculin test manufactured by Pasteur Merieux of Lyon, France contains a device suitable for introduction of CIC-containing compositions.

[0346] The device (which is distributed in the U.S. by Connaught Laboratories, Inc. of Swiftwater, PA) consists of a plastic container having a syringe plunger at one end and a tine disk at the other. The tine disk supports a multiplicity of narrow diameter tines of a length which will just scratch the outermost layer of epidermal cells. Each of the tines in the MONO-VACC kit is coated with old tuberculin; in the present invention, each needle is coated with a pharmaceutical composition of a CIC formulation. Use of the device is preferably according to the manufacturer's written instructions included with the device product. Similar devices which can also be used in this embodiment are those which are currently used to perform allergy tests.

[0347] Another suitable approach to epidermal administration of CIC is by use of a chemical which irritates the outermost cells of the epidermis, thus provoking a sufficient immune response to attract APCs to the area. An example is a keratinolytic agent, such as the salicylic acid used in the commercially available topical depilatory creme sold by Noxema Corporation under the trademark NAIR. This approach can also be used to achieve epithelial administration in the mucosa. The chemical irritant can also be applied in conjunction with the mechanical irritant (as, for example, would occur if the MONO-VACC type tine were also coated with the chemical



irritant). The CIC can be suspended in a carrier which also contains the chemical irritant or coadministered therewith.

[0348] Parenteral routes of administration include but are not limited to electrical (iontophoresis) or direct injection such as direct injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection. Formulations of CIC suitable for parenteral administration are generally formulated in USP water or water for injection and may further comprise pH buffers, salts bulking agents, preservatives, and other pharmaceutically acceptable excipients. CICs for parenteral injection may be formulated in pharmaceutically acceptable sterile isotonic solutions such as saline and phosphate buffered saline for injection.

[0349] Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. The invention includes formulations CIC suitable for gastrointestinal administration including, but not limited to, pharmaceutically acceptable powders, pills or liquids for ingestion and suppositories for rectal administration. As will be apparent to one of skill in the art, pills or suppositories will further comprise pharmaceutically acceptable solids, such as starch, to provide bulk for the composition.

[0350] Naso-pharyngeal and pulmonary administration include are accomplished by inhalation, and include delivery routes such as intranasal, transbronchial and transalveolar routes. The invention includes formulations of CIC suitable for administration by inhalation including, but not limited to, liquid suspensions for forming aerosols as well as powder forms for dry powder inhalation delivery systems. Devices suitable for administration by inhalation of CIC formulations include, but are not limited to, atomizers, vaporizers, nebulizers, and dry powder inhalation delivery devices.

[0351] The choice of delivery routes can be used to modulate the immune response elicited. For example, IgG titers and CTL activities were identical when an influenza virus vector was administered via intramuscular or epidermal (gene gun)

routes; however, the muscular inoculation yielded primarily IgG2a, while the epidermal route yielded mostly IgG1. Pertmer et al. (1996) *J. Virol.* 70:6119-6125. Thus, one skilled in the art can take advantage of slight differences in immunogenicity elicited by different routes of administering the immunomodulatory oligonucleotides of the present invention.

[0352] The above-mentioned compositions and methods of administration are meant to describe but not limit the methods of administering the formulations of CIC of the invention. The methods of producing the various compositions and devices are within the ability of one skilled in the art and are not described in detail here.

[0353] Analysis (both qualitative and quantitative) of the immune response to CIC can be by any method known in the art, including, but not limited to, measuring antigen-specific antibody production (including measuring specific antibody +subclasses), activation of specific populations of lymphocytes such as CD4+ T cells, NK cells or CTLs, production of cytokines such as IFN- $\gamma$ , IFN- $\alpha$ , IL-2, IL-4, IL-5, IL-10 or IL-12 and/or release of histamine. Methods for measuring specific antibody responses include enzyme-linked immunosorbent assay (ELISA) and are well known in the art. Measurement of numbers of specific types of lymphocytes such as CD4+ T cells can be achieved, for example, with fluorescence-activated cell sorting (FACS). Cytotoxicity and CTL assays can be performed for instance as described in Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523 and Cho et al. (2000). Cytokine concentrations can be measured, for example, by ELISA. These and other assays to evaluate the immune response to an immunogen are well known in the art. See, for example, SELECTED METHODS IN CELLULAR IMMUNOLOGY (1980) Mishell and Shiigi, eds., W.H. Freeman and Co.

[0354] Preferably, a Th1-type response is stimulated, *i.e.*, elicited and/or enhanced. With reference to the invention, stimulating a Th1-type immune response can be determined *in vitro* or *ex vivo* by measuring cytokine production from cells treated with a CIC as compared to control cells not treated with CIC. Methods to

determine the cytokine production of cells include those methods described herein and any known in the art. The type of cytokines produced in response to CIC treatment indicate a Th1-type or a Th2-type biased immune response by the cells. As used herein, the term "Th1-type biased" cytokine production refers to the measurable increased production of cytokines associated with a Th1-type immune response in the presence of a stimulator as compared to production of such cytokines in the absence of stimulation. Examples of such Th1-type biased cytokines include, but are not limited to, IL-2, IL-12, IFN- $\gamma$  and IFN- $\alpha$ . In contrast, "Th2-type biased cytokines" refers to those associated with a Th2-type immune response, and include, but are not limited to, IL-4, IL-5, and IL-13. Cells useful for the determination of CIC activity include cells of the immune system, primary cells isolated from a host and/or cell lines, preferably APCs and lymphocytes, even more preferably macrophages and T cells.

[0355] Stimulating a Th1-type immune response can also be measured in a host treated with a CIC can be determined by any method known in the art including, but not limited to: (1) a reduction in levels of IL-4 or IL-5 measured before and after antigen-challenge; or detection of lower (or even absent) levels of IL-4 or IL-5 in a CIC treated host as compared to an antigen-primed, or primed and challenged, control treated without CIC; (2) an increase in levels of IL-12, IL-18 and/or IFN ( $\alpha$ ,  $\beta$  or  $\gamma$ ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN ( $\alpha$ ,  $\beta$  or  $\gamma$ ) in a CIC treated host as compared to an antigen-primed or, primed and challenged, control treated without CIC; (3) "Th1-type biased" antibody production in a CIC treated host as compared to a control treated without CIC; and/or (4) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower (or even absent) levels of antigen-specific IgE in a CIC treated host as compared to an antigen-primed, or primed and challenged, control treated without CIC. A variety of these determinations can be made by measuring cytokines made by APCs and/or lymphocytes, preferably macrophages and/or T cells,

in vitro or ex vivo using methods described herein or any known in the art. Some of these determinations can be made by measuring the class and/or subclass of antigen-specific antibodies using methods described herein or any known in the art.

**[0356]** The class and/or subclass of antigen-specific antibodies produced in response to CIC treatment indicate a Th1-type or a Th2-type biased immune response by the cells. As used herein, the term "Th1-type biased" antibody production refers to the measurable increased production of antibodies associated with a Th1-type immune response (*i.e.*, Th1-associated antibodies). One or more Th1 associated antibodies may be measured. Examples of such Th1-type biased antibodies include, but are not limited to, human IgG1 and/or IgG3 (see, *e.g.*, Widhe et al. (1998) *Scand. J. Immunol.* 47:575-581 and de Martino et al. (1999) *Ann. Allergy Asthma Immunol.* 83:160-164) and murine IgG2a. In contrast, "Th2-type biased antibodies" refers to those associated with a Th2-type immune response, and include, but are not limited to, human IgG2, IgG4 and/or IgE (see, *e.g.*, Widhe et al. (1998) and de Martino et al. (1999)) and murine IgG1 and/or IgE.

**[0357]** The Th1-type biased cytokine induction which occurs as a result of administration of CIC produces enhanced cellular immune responses, such as those performed by NK cells, cytotoxic killer cells, Th1 helper and memory cells. These responses are particularly beneficial for use in protective or therapeutic vaccination against viruses, fungi, protozoan parasites, bacteria, allergic diseases and asthma, as well as tumors.

**[0358]** In some embodiments, a Th2 response is suppressed. Suppression of a Th2 response may be determined by, for example, reduction in levels of Th2-associated cytokines, such as IL-4 and IL-5, as well as IgE reduction and reduction in histamine release in response to allergen.

## V. Kits of the Invention

**[0359]** The invention provides kits. In certain embodiments, the kits of the invention comprise one or more containers comprising a CIC. The kits may further comprise a suitable set of instructions, generally written instructions, relating to the use of the CIC for the intended treatment (*e.g.*, immunomodulation, ameliorating symptoms of an infectious disease, increasing IFN- $\gamma$  levels, increasing IFN- $\alpha$  levels, or ameliorating an IgE-related disorder).

**[0360]** The kits may comprise CIC packaged in any convenient, appropriate packaging. For example, if the CIC is a dry formulation (*e.g.*, freeze dried or a dry powder), a vial with a resilient stopper is normally used, so that the CIC may be easily resuspended by injecting fluid through the resilient stopper. Ampoules with non-resilient, removable closures (*e.g.*, sealed glass) or resilient stoppers are most conveniently used for liquid formulations of CIC. Also contemplated are packages for use in combination with a specific device, such as an inhaler, nasal administration device (*e.g.*, an atomizer) or an infusion device such as a minipump.

**[0361]** The instructions relating to the use of CIC generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers of CIC may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine-readable instructions (*e.g.*, instructions carried on a magnetic or optical storage disk) are also acceptable.

**[0362]** In some embodiments, the kits further comprise an antigen (or one or more antigens), which may or may not be packaged in the same container (formulation) as the CIC(s). Antigen have been described herein.

**[0363]** In certain embodiments, the kits of the invention comprise a CIC in the form of a CIC/microcarrier complex (CIC/MC) and may further comprise a set of instructions, generally written instructions, relating to the use of the CIC/MC

complex for the intended treatment (*e.g.*, immunomodulation, ameliorating symptoms of an infectious disease, increasing IFN- $\gamma$  levels, increasing IFN- $\alpha$  levels, or ameliorating an IgE-related disorder).

**[0364]** In some embodiments, kits of the invention comprise materials for production of CIC/MC complex generally include separate containers of CIC and MC, although in certain embodiments materials for producing the MC are supplied rather than preformed MC. The CIC and MC are preferably supplied in a form which allows formation of CIC/MC complex upon mixing of the supplied CIC and MC. This configuration is preferred when the CIC/MC complex is linked by non-covalent bonding. This configuration is also preferred when the CIC and MC are to be crosslinked via a heterobifunctional crosslinker; either CIC or the MC is supplied in an "activated" form (*e.g.*, linked to the heterobifunctional crosslinker such that a moiety reactive with the CIC is available):

**[0365]** Kits for CIC/MC complexes comprising a liquid phase MC preferably comprise one or more containers including materials for producing liquid phase MC. For example, a CIC/MC kit for oil-in-water emulsion MC may comprise one or more containers containing an oil phase and an aqueous phase. The contents of the container are emulsified to produce the MC, which may be then mixed with the CIC, preferably a CIC which has been modified to incorporate a hydrophobic moiety. Such materials include oil and water, for production of oil-in-water emulsions, or containers of lyophilized liposome components (*e.g.*, a mixture of phospholipid, cholesterol and a surfactant) plus one or more containers of an aqueous phase (*e.g.*, a pharmaceutically-acceptable aqueous buffer).

## VI. Examples

**[0366]** The following Examples are provided to illustrate, but not limit, the invention.

*Example 1: Structure of Polynucleotides and Chimeric Compounds*

**[0367]** Table 2 shows the structures of polynucleotides and chimeric molecules referred to in the Examples. "HEG" is a hexa(ethylene glycol) spacer moiety; "TEG" is triethylene glycol; "C3" is a propyl spacer moiety; "C4" is a butyl spacer; "C6" is a hexyl spacer; "C12" is a dodecyl spacer; "HME" is 2-hydroxymethylethyl; "abasic" or "ab" is 1'2'-dideoxyribose. Other spacers are described in this specification and in the figures.

**[0368]** Except where noted in Table 2 or in specific examples, all nucleotide linkages and linkages between nucleic acid moieties and spacer moieties are phosphorothioate ester. For example, in CICs comprising compound (multiple subunits) spacer moieties with multiple HEG or C3 units (e.g., C-13, C-14, C-15, C-15, C-91, C-92, C-36, C-37, and C-38) the C3 or HEG units are linked with a phosphorothioate linker. Similarly, the branched CICs shown (e.g., C-93, C-94, C-95, C-96, C-97, C-98, C-100, C-101, C-103, C-104, C-121, C-122, C-123, C-124, C-125, C-126, C-127, C-129, C-130) comprise phosphorothioate linkers between the branching subunit and the linear subunit of the spacer. Other branched CICs shown (e.g., C-26, C-99, C-102, C-105, and C-137) are prepared by conjugation strategies and have linking groups as described in the Examples.

**[0369]** Table 2 also includes CICs (e.g., C-128, C-106- C-113 ) with an end linking group (e.g.,  $\text{HS}(\text{CH}_2)_6$  - and  $\text{HO}(\text{CH}_2)_6\text{SS}(\text{CH}_2)_6$ ) used to link these molecules with branched spacer moieties to create branched CICs. See, e.g., Example 18. These linking groups are connected to the CIC with a phosphorothioate linkage.

TABLE 2

## TEST COMPOUNDS AND POLYNUCLEOTIDES

Compound Designation Number(s)	Structure
P-1	5'-TCGTCGA-3'
P-2	5'-TCGTCCG-3'
P-3	5'-ACGTTCCG-3'
P-4	5'-AGATGAT-3'
P-5	5'-ATCTCGA-3'
P-6	5'-TGA CTG TGA ACG TTC GAG ATG A-3' (SEQ ID NO:2)
P-7	5'-TGA CTG TGA ACC TTA GAG ATG A-3' (SEQ ID NO:3)
P-8	5'-TGA CTG TGA ACG TTC GAG ATG A-3' (SEQ ID NO:136)
P-9	5'-CTGTGAACGTTTCGAGATG-3' (SEQ ID NO:83)
P-10	5'-TCGTGCAACGTTTCGAGATG-3' (SEQ ID NO:41)
P-11	5'-AACGTT-3'
P-12	5'-TCGTCCG-3'
P-13	5'-TCGAGAT-3'
P-14	5'-TCGACGT-3'
P-15	HO(CH <sub>2</sub> ) <sub>6</sub> SS(CH <sub>2</sub> ) <sub>6</sub> -5'-TGA CTG TGA ACG TTC GAG ATG A-3' (SEQ ID NO:137)
P-16	HS(CH <sub>2</sub> ) <sub>6</sub> -5'-TGA CTG TGA ACG TTC GAG ATG A-3' (SEQ ID NO:138)
P-17	5'-TCGAACGTTTCGA-3' (SEQ ID NO:155)
M-1	5'-TGCTGC-3'-HEG-5'-AGCTTGC-3'-HEG-5'-AGATGAT-3'
M-2	5'-TCCTCCA-3'-HEG-5'-ACCTTAG-3'-HEG-5'-AGATGAT-3'
M-3	(5'-TAGTCAT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-AACCTTC-3'
M-17	(5'-TAGTCAT-3'-HEG) <sub>2</sub> -symmetrical doubler-HEG-5'-TAGTCAT-3'
M-18	(5'-TAGTCAT-3'-HEG) <sub>3</sub> -trebler-HEG-5'-TAGTCAT-3'
M-19	(5'-TAGTCAT-3'-TEG) <sub>2</sub> -glycerol-TEG-5'-TAGTCAT-3'
M-20	(5'-TAGTCAT-3'-C3) <sub>2</sub> -glycerol-C3-5'-TAGTCAT-3'
M-21	5'-TCCTCCA-3'-HEG-5'-ACCTTAG-3'-HEG-5'-AGATGAT-C <sub>6</sub> NH <sub>2</sub>
M-22	(5'-TAGTCAT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-AACCTTC-3'-C <sub>6</sub> NH <sub>2</sub>
C-8	5'-TCGTCGA-3'-HEG-5'-ACGTTCCG-3'-HEG-5'-AGATGAT-3'
C-9	5'-TCGTCGA-3'-C3-5'-ACGTTCCG-3'-C3-5'-AGATGAT-3'
C-10	5'-TCGTCCG-3'-HEG-5'-ACGTTCCG-3'-HEG-5'-AGATGAT-3'
C-11	5'-TCGTCCG-3'-C3-5'-ACGTTCCG-3'-C3-5'-AGATGAT-3'
C-12	(5'-TCGTCGA-3') <sub>2</sub> -glycerol-3'-AGCTGCT-5'
C-13	5'-TCGTCCG-3'-(C3) <sub>15</sub> -5'-T-3'
C-14	5'-TCGTCCG-3'-(HME) <sub>15</sub> -5'-T-3'
C-15	5'-TCGTCCG-3'-(TEG) <sub>8</sub> -5'-T-3'
C-16	5'-TCGTCCG-3'-(HEG) <sub>4</sub> -5'-T-3'
C-17	5'-TCGTCCG-3'-C4-5'-ACGTTCCG-3'-C4-5'-AGATGAT-3'
C-18	5'-TCGTCCG-3'-TEG-5'-ACGTTCCG-3'-TEG-5'-AGATGAT-3'
C-19	5'-TCGTCCG-3'-C12-5'-ACGTTCCG-3'-C12-5'-AGATGAT-3'
C-20	5'-TCGTCCG-3'-abasic-5'-ACGTTCCG-3'-abasic-5'-AGATGAT-3'
C-21	5'-TCGTCGA-3'-HEG-5'-TCGTCGA-3'-HEG-5'-TCGTCGA-3'
C-22	5'-TCGTCCG-3'-HEG-5'-TCGTCCG-3'-HEG-5'-TCGTCCG-3'



C-23	5'-TCGTTCG-3'-HEG-5'-AACGTT-3'-HEG-5'-AGATGAT-3'
C-24	5'-ACGTTTCG-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-AGATGAT-3'
C-25	5'-TCGTTCG-3'-HME-5'-ACGTTTCG-3'-HME-5'-AGATGAT-3'
C-26	(5'-TCGTTCG-3') <sub>4</sub> -R where R = Starburst Dendrimer® (See Ex. 18)
C-27	(5'-TCGTTCG-3') <sub>2</sub> -glycerol-5'-AACGTT-3'
C-28	(5'-TCGTTCG-3') <sub>2</sub> -glycerol-5'-TCGTTCG-3'
C-29	5'-TCGTTCG-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-AGATGAT-3'-TEG
C-30	HEG-5'-TCGTTCG-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-AGATGAT-3'-TEG
C-31	HEG-5'-TCGTTCG-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-AGATGAT-3'-TEG (phosphodiester linkages)
C-32	5'-TCG-3'-HEG-5'-TCG-3'-HEG-5'-TCG-3'-HEG-5'-TCG-3'-HEG-5'-TCG-3'-HEG-5'-TCG-3'
C-33	5'-TCGTTCG-3'-C3-5'-TCGTTCG-3'-C3-5'-TCGTTCG-3' all phosphorothioate linkages
C-34	HS(CH <sub>2</sub> ) <sub>6</sub> -5'-TCGTTCG-3'-C3-5'-ACGTTTCG-3'-C3-5'-AGATGAT-3'
C-35	<div style="display: flex; align-items: center; justify-content: center;"> <div style="text-align: center;"> 5'-TCGTTCG-3'    5'-AACGTT-3' </div> <div style="margin: 0 10px;"> \   / </div> <div style="text-align: center;"> glycerol-5'-AGATGAT-3' </div> </div>
C-36	5'-TCGTTCG-3'-(HEG) <sub>6</sub> -5'-TCGTTCG-3' (phosphodiester linkages)
C-37	5'-TCGTTCG-3'-(HEG) <sub>4</sub> -3'-AGCTGCT-5'
C-38	5'-TCGTTCG-3'-(HEG) <sub>4</sub> -5'-TCGTTCG-3'
C-39	5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'
C-40	5'-TCGTTCG-3'-HEG-5'-TCG-3'
C-41	5'-TCGTTTT-3'-HEG-5'-TCGTTTT-3'-HEG-5'-TCGTTTT-3'
C-42	5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'
C-43	5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'
C-44	5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'-HEG-5'-TCGTTCG-3'
C-45	5'-TCGAGAT-3'-HEG-5'-TCGAGAT-3'-HEG-5'-TCGAGAT-3'
C-46	5'-TTCGTTTT-3'-HEG-5'-TTCGTTTT-3'-HEG-5'-TTCGTTTT-3'
C-47	5'-TCGTTCG-3'-HEG-5'-TGCTGTT-3'-HEG-5'-TGCTGTT-3'
C-48	5'-TCGTTCG-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-TCGTTCG-3'
C-49	5'-TCGTTCG-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-GGGGGG-3'
C-50	5'-TCGAACG-3'-HEG-5'-TCGAACG-3'-HEG-5'-TCGAACG-3'
C-51	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-HEG-5'-TCGACGT-3'
C-52	5'-CGTTTCG-3'-HEG-5'-CGTTTCG-3'-HEG-5'-CGTTTCG-3'
C-53	5'-TGACTGTGA-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-AGATGAT-3'
C-54	5'-TCGTTCG-3'-HEG-5'-AACGTT-3'-HEG-5'-AGATGAT-3'
C-55	5'-TCGTTCG-3'-HEG-5'-AACGTT-3'-HEG-5'-TCGTTCG-3'
C-56	5'-TCGTTCG-3'-HEG-5'-AGATGAT-3'-HEG-5'-ACGTTTCG-3'
C-57	5'-ACGTTTCG-3'-HEG-5'-TCGTTCG-3'-HEG-5'-AGATGAT-3'
C-58	5'-ACGTTTCG-3'-HEG-5'-AGATGAT-3'-HEG-5'-TCGTTCG-3'
C-59	5'-AGATGAT-3'-HEG-5'-TCGTTCG-3'-HEG-5'-ACGTTTCG-3'
C-60	5'-AGATGAT-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-TCGTTCG-3'
C-61	5'-TCCATTT-3'-HEG-5'-AACGTT-3'-HEG-5'-TGACGTT-3'
C-62	5'-TGACGTT-3'-HEG-5'-AACGTT-3'-HEG-5'-TCCATTT-3'

C-63	5'-TCGACTC-3'-HEG-5'-TCGAGCG-3'-HEG-5'-TTCTCTT-3'
C-64	5'-CTGTGAACGTTTCGAGATG-3' (SEQ ID NO:83)-HEG-5'-CTGTGAACGTTTCGAGATG-3' (SEQ ID NO:83)
C-65	5'-TCGTCTGA-3'-HEG-5'-TCGTCTGA-3'-HEG-3'-AGCTGCT-5'
C-66	5'-TCGTCTGAACGTTTCGAGATG-3' (SEQ ID NO:41)-HEG-5'-TCGTCTGAACGTTTCGAGATG-3' (SEQ ID NO:41)
C-67	5'-TCGTCTGAACGTTTCGAGATG-3' (SEQ ID NO:41)-HEG-3'-GTAGAGCTTGCAAGCTGCT-5' (SEQ ID NO:41)
C-68	5'-TCG-3'-HEG-5'-T-3'
C-69	5'-TCGAT-3'-HEG-5'-TCGAT-3'-HEG-5'-TCGAT-3'-HEG-5'-TCGAT-3'
C-70	5'-TCGTCTGA-3'-HEG-5'-TCGTCTGA-3'-HEG-5'-AACGTTC-3'-HEG-5'-AGAT-3'
C-71	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-HEG-5'-TCGACGT-3'
C-72	5'-TCCTCCA-3'-HEG-5'-ACCTTAG-3'-HEG-5'-AGATGAT-3' (no CG)
C-73	5'-ACGTCTGA-3'-HEG-5'-ACGTCTGA-3'-HEG-5'-ACGTCTGA-3'
C-74	5'-TCGATTT-3'-HEG-5'-TCGATTT-3'-HEG-5'-TCGATTT-3'
C-75	5'-TTCGATT-3'-HEG-5'-TTCGATT-3'-HEG-5'-TTCGATT-3'
C-76	5'-TTTCGAT-3'-HEG-5'-TTTCGAT-3'-HEG-5'-TTTCGAT-3'
C-77	5'-TTTTCTGA-3'-HEG-5'-TTTTCTGA-3'-HEG-5'-TTTTCTGA-3'
C-78	5'-TCGCTTT-3'-HEG-5'-TCGCTTT-3'-HEG-5'-TCGCTTT-3'
C-79	5'-TCGGTTT-3'-HEG-5'-TCGGTTT-3'-HEG-5'-TCGGTTT-3'
C-80	5'-ACGATTT-3'-HEG-5'-ACGATTT-3'-HEG-5'-ACGATTT-3'
C-81	5'-ATCGAT-3'-HEG-5'-ATCGAT-3'-HEG-5'-ATCGAT-3'
C-82	5'-ATCGATT-3'-HEG-5'-ATCGATT-3'-HEG-5'-ATCGATT-3'
C-83	5'-AACGTT-3'-HEG-5'-AACGTT-3'-HEG-5'-AACGTT-3'
C-84	5'-GsGs-3'-C3-5'-TGC-3'-C3-5'-ATCGAT-3'-C3-5'-GCA-3'-C3-5'-GGsGsGsGsG-3' (s = phosphorothioate linkages, otherwise linkages are phosphodiester)
C-85	5'-GsGs-3'-C3-5'-TCGTGC-3'-C3-5'-ATCGAT-3'-C3-5'-GCACGA-3'-C3-5'-GGsGsGsGsG-3' (s = phosphorothioate linkages, otherwise linkages are phosphodiester)
C-86	5'-TGCTGCA-3'-C3-5'-AGCTTGC-3'-C3-5'-AGATGAT-3' (No CG)
C-87	5'-GsGsGsGs-3'-C3-5'-ATCGAT-3'-C3-5'-TGATGCATCA-3'-C3-5'-ATCGAT-3'-C3-5'-GsGsGsGsG-3' (s = phosphorothioate linkages, otherwise linkages are phosphodiester) (TGATGCATCA is SEQ ID NO:105)
C-88	5'-TCCA-3'-C3-5'-TGACGTT-3'-C3-5'-CCTGATGCT-3'
C-89	5'-TGAAGTGA-3'-C3-5'-ACGTTTCG-3'-C3-AGATGAT-3'
C-90	5'-TCGTCTGA-3'-C3-5'-TCGTCTGA-3'-C3-5'-TCGTCTGA-3'
C-91	5'-TCG-3'-(ab) <sub>3</sub> -5'-T-3'
C-92	(ab)-5'-TCG-3'-(ab) <sub>2</sub> -5'-T-3'
C-93	(5'-TCGTCTGA-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGTCTGA-3' (phosphodiester)
C-94	(5'-TCGTCTGA-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGTCTGA-3'
C-95	(5'-TCGTCTGA-3'-HEG) <sub>2</sub> -glycerol-HEG-3'-AGCTGCT-5'
C-96	(5'-TCGTCTGA-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-AACGTTC-3'
C-97	(5'-TCGTCTGA-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-AACGTTC-3'-HEG-5'-TCGA-3'
C-98	(5'-TCGTCTGA-3'-HEG) <sub>3</sub> -trebler-HEG-5'-AACGTTC-3'-HEG-5'-TCGA-3'

C-99	TMEA-(5'-TGA CTGTGAACGTTTCGAGATGA-3') <sub>3</sub> (SEQ ID NO:139) (See Ex. 23)
C-100	(5'-TCGTCTGA-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-AACGTTT-3'-HEG-5'-TCGACGT-3'
C-101	(5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGACGT-3'
C-102	Starburst Dendrimer®-(5'-TGA CTGTGAACGTTTCGAGATGA-3') <sub>x</sub> (X range = 3-16) (SEQ ID NO:2) (See Ex. 24)
C-103	(5'-TCGTCTGA-3'-TEG) <sub>2</sub> -glycerol-TEG-5'-TCGTCTGA-3'
C-104	(5'-TCGTCTGA-3'-C3) <sub>2</sub> -glycerol-C3-5'-TCGTCTGA-3'
C-105	TMEA-(S-(CH <sub>2</sub> ) <sub>3</sub> -3'-TAGTAGA-5'-HEG-3'-GCTTGCA-5'-HEG-3'-AGCTGCT-5') <sub>3</sub> (See Ex. 23)
C-106	HO(CH <sub>2</sub> ) <sub>6</sub> SS(CH <sub>2</sub> ) <sub>6</sub> -5'-TGA CTGTGAACGTTTCGAGATGA-3' (SEQ ID NO:134)
C-107	HS(CH <sub>2</sub> ) <sub>6</sub> -5'-TGA CTGTGAACGTTTCGAGATGA-3' (SEQ ID NO:135)
C-110	HO(CH <sub>2</sub> ) <sub>6</sub> SS(CH <sub>2</sub> ) <sub>6</sub> -5'-TCGTCTG-3'-C3-5'-ACGTTCTG-3'-C3-5'-AGATGAT-3'
C-111	HS(CH <sub>2</sub> ) <sub>6</sub> -5'-TCGTCTG-3'-C3-5'-ACGTTCTG-3'-C3-5'-AGATGAT-3'
C-112	HO(CH <sub>2</sub> ) <sub>6</sub> SS(CH <sub>2</sub> ) <sub>6</sub> -5'-TCGTCTGA-3'-HEG-5'-ACGTTCTG-3'-HEG-5'-AGATGAT-3'
C-113	HS(CH <sub>2</sub> ) <sub>6</sub> -5'-TCGTCTGA-3'-HEG-5'-ACGTTCTG-3'-HEG-5'-AGATGAT-3'
C-114	5'-TCGTCTGA-3'-C3-5'-ACGTTCTG-3'-C3-5'-AGATGAT-3'-C3-(CH <sub>2</sub> ) <sub>3</sub> SS(CH <sub>2</sub> ) <sub>3</sub> OH
C-115	5'-TCGTCTGA-3'-C3-5'-ACGTTCTG-3'-C3-5'-AGATGAT-3'-C3-(CH <sub>2</sub> ) <sub>3</sub> SH
C-116	5'-TCGTCTGA-3'-HEG-5'-ACGTTCTG-3'-HEG-5'-AGATGAT-3'-(CH <sub>2</sub> ) <sub>3</sub> SS(CH <sub>2</sub> ) <sub>3</sub> OH
C-117	5'-TCGTCTGA-3'-HEG-5'-ACGTTCTG-3'-HEG-5'-AGATGAT-3'-(CH <sub>2</sub> ) <sub>3</sub> SH
C-118	5'-TCGTCTGA-3'-HEG-C3-5'-ACGTTCTG-3'-HEG-5'-AGATGAT-3'
C-119	5'-TCGA-3'-HEG-5'-TCGA-3'-HEG-5'-TCGA-3'-HEG-5'-TCGA-3'-HEG-5'-TCGA-3'
C-120	5'-TCGTCTG-3'-C6-5'-ACGTTCTG-3'-C6-5'-AGATGAT-3'
C-121	(5'-AACGTT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-AACGTT-3'
C-122	(5'-TCAACGTT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCAACGTT-3'
C-123	(5'-TCGTCTGA-3'-HEG-HEG) <sub>2</sub> -glycerol-HEG-HEG-5'-TCGTCTGA-3'
C-124	(5'-TCGACGT-3'-HEG) <sub>2</sub> -symmetrical doubler-HEG-5'-TCGACGT-3'
C-125	(5'-TCGACGT-3'-HEG) <sub>3</sub> -trebler-HEG-5'-TCGACGT-3'
C-126	((5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGACGT-3'
C-127	(5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-AACGTTT-3'
C-128	HO(CH <sub>2</sub> ) <sub>6</sub> SS(CH <sub>2</sub> ) <sub>6</sub> -5'-TCGTCTGA-3'-C3-5'-ACGTTCTG-3'-C3-5'-AGATGAT-3'
C-129	((5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-HEG) <sub>2</sub> -glycerol-HEG-5'-T-3'
C-130	(5'-TCGACGT-3'-HEG) <sub>3</sub> -trebler-HEG-5'-T-3'
C-131	5'-TCGTCTGA-3'-C4-5'-ACGTTCTG-3'-C4-5'-AGATGAT-3'
C-132	5'-TCGTCTGA-3'-C6-5'-ACGTTCTG-3'-C6-5'-AGATGAT-3'
C-133	5'-TCGTCTGA-3'-TEG-5'-ACGTTCTG-3'-TEG-5'-AGATGAT-3'
C-134	5'-TCGTCTGA-3'-PEG-5'-ACGTTCTG-3'-PEG-5'-AGATGAT-3' [PEG=(CH <sub>2</sub> CH <sub>2</sub> O) <sub>45</sub> ]
C-135	5'-TCGACGT-3'-HEG-(CH <sub>2</sub> ) <sub>3</sub> SS(CH <sub>2</sub> ) <sub>3</sub> OH
C-136	5'-TCGACGT-3'-HEG-(CH <sub>2</sub> ) <sub>3</sub> SH

C-137	(5'-TCGACGT-3'-HEG) <sub>x</sub> -Ficoll <sub>400</sub> (X range = 150-250, ave. 185) See example 49
C-138	Ficoll-(5'-P-6) <sub>156</sub>
C-139	bPEG-(5'-P-6) <sub>2-4</sub>
C-140	(5'-TCGACGT-3'-HEG) <sub>2-4</sub> -bPEG
C-141	(5'-TCGACGT-3'-HEG) <sub>3</sub> -TMEA
C-142	(5'-TCGTCGT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGTCGT-3'
C-143	(5'-TCGTTTT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGTTTT-3'
C-144	(5'-TCGAACG-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGAACG-3'
C-145	(5'-TCGATCG-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGATCG-3'
C-146	(5'-TCGAGAT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGAGAT-3'
C-147	(5'-TCGATTT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGATTT-3'
C-148	(5'-TCGACGA-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGACGA-3'
C-149	(5'-TCGAGCT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGAGCT-3'
C-150	(5'-TCGAATT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGAATT-3'
C-151	5'-TCGTCGA-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-AGATGAT-3'-C <sub>6</sub> NH <sub>2</sub>
C-152	(5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGACGT-3'-C <sub>6</sub> NH <sub>2</sub>
C-153	(5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGTCGA-3'
C-154	(5'-TCGTCGA-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGACGT-3'
C-155	5'-TCGTCGA-3'-HEG glycerol-HEG-5'-TCGTCGA-3' 5'-TCGACGT-3'-HEG
C-156	(5'-TCGCTCG-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGCTCG-3'
C-157	(5'-TCGGTCG-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGGTCG-3'
C-158	(5'-TCGTTTCG-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGTTTCG-3'
C-159	5'-TGCGTGTAACGTTACACGCA-3' (SEQ ID NO:114)-HEG-5'-TGCGTGTAACGTTACACGCA-3' (SEQ ID NO:114)
C-160	5'-TGCGTGTAACGTTACACGCA-3' (SEQ ID NO:114)-HEG-5'-TGCGTGTAACGTTACAC-3' (SEQ ID NO:114)
C-161	(5'-CTGAACGTTTCAG-3' (SEQ ID NO:104)-HEG) <sub>2</sub> -glycerol-HEG-5'-CTGAACGTTTCAG-3' (SEQ ID NO:104)
C-162	(5'-CTGAACGTTTCAG-3' (SEQ ID NO:104)-HEG) <sub>2</sub> -glycerol-HEG-3'-GACTTGCAAGTC-5' (SEQ ID NO:104)
C-163	(5'-CTGAACGTTTCAG (SEQ ID NO:104) -3'-HEG) <sub>3</sub> -trebler-HEG-5'-T-3'
C-164	(5'-CTGAACGTTTCAG (SEQ ID NO:104) -3'-HEG) <sub>3</sub> -trebler-HEG-5'-T-3' (all phosphodiester)
C-165	(5'-TGCGTGTAACGTTACACGCA-3' (SEQ ID NO:114)-HEG) <sub>2</sub> -glycerol-HEG-5'-T-3'
C-166	(5'-TGCGTGTAACGTTACACGCA-3') <sub>2</sub> (SEQ ID NO:114)-glycerol-HEG-5'-T-3' (all phosphodiester)
C-167	(5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-HEG-5'-TTGGCCAAGCTTGGCCAA-3' (SEQ ID NO:116)
C-168	5'-TCGTCGA-3'-HEG-(gly(HEG-3'-TGCAGCT-5'))-HEG) <sub>3</sub> -5'-TCGAACG-3'
C-169	5'-TCGTCGA-3'-HEG-(gly(HEG-3'-TGCAGCT-5'))-5'-TTTTT-3') <sub>3</sub> -HEG-5'-TCGAACG-3'

C-170	(5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-HEG-glycerol-(HEG-3'-TGCAGCT-5') <sub>2</sub>
C-171	(5'-TCGACGT-3'-HEG) <sub>2</sub> -glycerol-5'-TTTTT-3'-glycerol-(HEG-3'-TGCAGCT-5') <sub>2</sub>
C-172	5'-TCGTTTGAACGTTCCGAACGA-3' (SEQ ID NO:153) -HEG- 5'-TCGTTTGAACGTTCCGAACGA-3' (SEQ ID NO:154)
C-173	(5'-TCGAACGTTTGA-3' (SEQ ID NO:155) -HEG) <sub>2</sub> -glycerol-HEG-5'-TCGAACGTTTGA-3' (SEQ ID NO:155)
C-174	(5'-TCGAACGTTTGA-3' (SEQ ID NO:155) -HEG) <sub>2</sub> -glycerol-HEG-3'-AGCTTGCAAGCT-5' (SEQ ID NO:155)
C-175	(5'-TCGAACGTTTGA-3' (SEQ ID NO:155) -HEG) <sub>3</sub> -trebler-HEG-5'-T-3'
C-176	5'-TCGTTTGAACGTTCCGAACGA-3' (SEQ ID NO:153) -HEG-5'-TCGTTTGAACGTTTGA-3' (SEQ ID NO:156)
C-177	(5'-TCGTTTGAACGTTCCGAACGA-3' (SEQ ID NO:153) -HEG) <sub>2</sub> -glycerol-HEG-5'-T-3'
C-178	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-HEG-5'-TTGGCCAAGCTTGGCCAA (SEQ ID NO:116)
C-179	5'-TCGTCTGA-3'-HEG-5'-TCGTCTGA-3'-HEG-5'-ACGTTTCG-3'
C-180	5'-TCGTCTGA-3'-TEG-5'-TCGTCTGA-3'-TEG-5'-ACGTTTCG-3'
C-181	5'-TCGTCTGA-3'-C6-5'-TCGTCTGA-3'-C6-5'-ACGTTTCG-3'
C-182	5'-TCGTCTGA-3'-HEG-5'-ACGTTTCG-3'-HEG-5'-TCGAGAT-3'
C-183	5'-TCGACGT-3'-TEG-5'-TCGTCTGA-3'-TEG-5'-ACGTTTCG-3'
C-184	5'-TCGTCTGA-3'-TEG-5'-TCGACGT-3'-TEG-5'-ACGTTTCG-3'
C-185	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-HEG-5'-TCGTCTGA-3'
C-186	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-C3-5'-ACGTTTCG-3'
C-187	5'-TCGAACGTTTGA-3' (SEQ ID NO:155) -HEG-5'-TCGAACGTTTGA-3' (SEQ ID NO:155)
C-188	(5'-TCGAACGTTTGA-3 (SEQ ID NO:155) '-HEG) <sub>2</sub> -glycerol-HEG-5'-T-3'
C-189	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-HEG-5'-AACGTTTC-3'
C-190	5'-TCGACGT-3'-HEG-5'-AACGTTTC-3'-HEG-5'-TCGACGT-3'
C-191	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-HEG-5'-AACGTTTC-3'
C-192	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-HEG-5'-AACGTTTC-3'-HEG-5'-TCGACGT-3'
C-193	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-TEG-5'-ACGTTTCG-3'
C-194	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-C6-5'-ACGTTTCG-3'
C-195	5'-TCGACGT-3'-HEG-5'-TCGACGT-3'-C4-5'-ACGTTTCG-3'
C-196	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGACGT-3'-HEG-5'-AACGTTTC-3'
C-197	5'-TCGATCG-3'-HEG-5'-TCGATCG-3'-HEG-5'-TCGATCG-3'
C-198	(5'-TCGGCGC-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGGCGC-3'
C-199	(5'-TCGCCGG-HEG) <sub>2</sub> -glycerol-HEG-5'-TCGCCGG-3'
C-200	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-C4-5'-ACGTTTCG-3'
C-201	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-5'-TCG-3'-C4-5'-TCGACGT-3'
C-202	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-HEG-5'-ACTTAGAGGTTTCACTAGG-3' (SEQ ID NO:157)
C-203	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-HEG-5'-CCTACTGAACCTCTAAGT-3' (SEQ ID NO:158)

C-204	(5'-AACGTTC-HEG) <sub>2</sub> -glycerol-5'-AACGTTC-3'
C-205	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-5'-GACGTTC-3'
C-206	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-5'-GACGTCC-3'
C-207	(5'-TCGACGT-HEG) <sub>2</sub> -glycerol-5'-AGCGCTC-3'
C-208	(5'-TCGTTTCG-HEG) <sub>2</sub> -glycerol-HEG-5'-ACTTAGAGGTTCACTAGG-3' (SEQ ID NO:157)
C-209	(5'-TCGTTTCG-HEG) <sub>2</sub> -glycerol-HEG-5'-CCTACTGAACCTCTAAGT-3' (SEQ ID NO:158)

*Example 2: Synthesis of a Chimeric Compound with a Linear Structure and Hexaethylene Glycol Spacers*

**[0370]** C-10, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are hexaethylene glycol (HEG), connected to the nucleic acid moieties via phosphorothioate linkages.

**[0371]** C-10: 5'-TCGTTCG-3'-HEG-5'-ACGTTCG-3'-HEG-5'-AGATGAT-3'

**[0372]** The C-10 molecule was synthesized by TriLink BioTechnologies (SanDiego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1 umol phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-hexaethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. (As will be apparent to the ordinarily skilled reader, the terms "nucleoside monomer" or "spacer moiety" are sometimes used herein, e.g., in the context of synthesis of CICs, to refer to the *precursor* reagents that when deprotected and linked to other components using synthetic methods such as those disclosed herein, give rise to the nucleic acid and nonnucleic acid moieties of the CICs.) The HEG spacer precursor was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and HEG spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "T" solid support
2. Synthesis of 5'-AGATGA-3' moiety
3. Addition of HEG spacer
4. Synthesis of 5'-ACGTTCG-3' moiety
5. Addition of HEG spacer

## 6. Synthesis of 5'-TCGTCG-3' moiety

[0373] The synthesis cycle consisted of a detritylation step, a coupling step (phosphoramidite monomer plus 1H-tetrazole), a capping step, a sulfurization step using 0.05 M 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent), and a final capping step. At the completion of assembly, the 'trityl-off' compound was cleaved from the controlled-pore glass and the bases were deprotected with concentrated aqueous ammonia at 58°C for 16 hours. The compound was purified by preparative polyacrylamide electrophoresis, desalted on a Sep-pak Plus cartridge (Waters, Milford, MA), and precipitated from 1 M aqueous sodium chloride with 2.5 volumes of 95% ethanol. The molecule was dissolved in Milli Q water and the yield was determined from the absorbance at 260 nm. Finally, the compound was lyophilized to a powder. The compound was characterized by capillary gel electrophoresis, electrospray mass spectrometry, and RP-HPLC to confirm composition and purity. An endotoxin content assay (LAL assay, Bio Whittaker) was also conducted, showing endotoxin levels were <5 EU/mg compound (i.e., essentially endotoxin free).

[0374] C-8, C-21, C-22, C-23, C-24, C-32 and M-1 and other linear HEG-CICs were synthesized analogously.

### *Example 3: Synthesis of a Chimeric Compound with a Linear Structure and Propyl Spacers*

[0375] C-11, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are propyl (C3), connected to the nucleic acid moieties via phosphorothioate linkages.

[0376] C-11: 5'-TCGTCG-3'-C3-5'-ACGTTTCG-3'-C3-5'-AGATGAT-3'

[0377] The C-11 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The nucleoside



monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-propyl-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The C3 spacer precursor was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and C3 spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "T" solid support
2. Synthesis of 5'-AGATGA-3' moiety
3. Addition of C3 spacer
4. Synthesis of 5'-ACGTTTCG-3' moiety
5. Addition of C3 spacer
6. Synthesis of 5'-TCGTCG-3' moiety

[0378] The synthesis, deprotection, workup, and analysis were performed as described in Example 2.

[0379] C-9 and other C3-containing CICs were synthesized analogously.

*Example 4: Synthesis of a Chimeric Compound with a Linear Structure and with Butyl Spacers*

[0380] C-17, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are butyl (C4), connected to the nucleic acid moieties via phosphorothioate linkages.

[0381] C-17: 5'-TCGTCG-3'-C4-5'-ACGTTTCG-3'-C4-5'-AGATGAT-3'

[0382] The C-17 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-butyl-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from ChemGenes, Ashland,

MA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The C4 spacer precursor was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and C4 spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "T" solid support
2. Synthesis of 5'-AGATGA-3' moiety
3. Addition of C4 spacer
4. Synthesis of 5'-ACGTTTCG-3' moiety
5. Addition of C4 spacer
6. Synthesis of 5'-TCGTTCG-3' moiety

[0383] The synthesis, deprotection, workup, and analysis were performed as described in Example 2.

*Example 5: Synthesis of a Chimeric Compound with a Linear Structure and Triethylene Glycol Spacers*

[0384] C-18, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are triethylene glycol (TEG), connected to the nucleic acid moieties via phosphorothioate linkages.

[0385] C-18: 5'-TCGTTCG-3'-TEG-5'-ACGTTTCG-3'-TEG-5'-AGATGAT-3'

[0386] The C-18 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-triethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The TEG spacer precursor was placed in an auxiliary

monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and TEG spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "T" solid support
2. Synthesis of 5'-AGATGA-3' moiety
3. Addition of TEG spacer
4. Synthesis of 5'-ACGTTCG-3' moiety
5. Addition of TEG spacer
6. Synthesis of 5'-TCGTCG-3' moiety

[0387] The synthesis, deprotection, workup, and analysis were performed as described in Example 2.

*Example 6: Synthesis of a Chimeric Compound with a Linear Structure and Dodecyl Spacers*

[0388] C-19, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are dodecyl (C12), connected to the nucleic acid moieties via phosphorothioate linkages.

[0389] C-19: 5'-TCGTCG-3'-C12-5'-ACGTTCG-3'-C12-5'-AGATGAT-3'

[0390] The C-19 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-dodecyl-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The C12 spacer precursor was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and C12 spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "T" solid support
2. Synthesis of 5'-AGATGA-3' moiety
3. Addition of C12 spacer
4. Synthesis of 5'-ACGTTCG-3' moiety
5. Addition of C12 spacer
6. Synthesis of 5'-TCGTCG-3' moiety

[0391] The synthesis, deprotection, workup, and analysis were performed as described in Example 2.

*Example 7: Synthesis of a Chimeric Compound with a Linear Structure and Abasic Spacers*

[0392] C-20, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are 1',2'-dideoxyribose (abasic), connected to the nucleic acid moieties via phosphorothioate linkages.

[0393] C-20: 5'-TCGTCG-3'-abasic-5'-ACGTTCG-3'-abasic-5'-AGATGAT-3'

[0394] The C-20 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1 umol phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursor, 5'-O-(4,4'-dimethoxytrityl)-1',2'-dideoxyribose-3'-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The abasic spacer precursor was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and abasic spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "T" solid support
2. Synthesis of 5'-AGATGA-3' moiety

3. Addition of abasic spacer
4. Synthesis of 5'-ACGTTCG-3' moiety
5. Addition of abasic spacer
6. Synthesis of 5'-TCGTCG-3' moiety

[0395] The synthesis, deprotection, workup, and analysis were performed as described in Example 2.

*Example 8: Synthesis of a Chimeric Compound with a Linear Structure and Hexaethylene Glycol and Triethylene Glycol Spacers*

[0396] C-29, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, the spacer moieties are hexaethylene glycol (HEG), connected to the nucleic acid moieties via phosphorothioate linkages, and the 3'-end group is triethylene glycol (TEG), connected to the nucleic acid moiety via a phosphorothioate linkage.

[0397] C-29: 5'-TCGTCG-3'-HEG-5'-ACGTTCG-3'-HEG-5'-AGATGAT-3'-TEG

[0398] The C-29 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The triethylene glycol-controlled-pore glass, used as the solid support for the synthesis, was from Glen Research (Sterling, VA). The nucleoside monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-hexaethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The HEG spacer was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and HEG spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a triethylene glycol solid support
2. Synthesis of 5'-AGATGAT-3' moiety
3. Addition of HEG spacer
4. Synthesis of 5'-ACGTTCG-3' moiety
5. Addition of HEG spacer
6. Synthesis of 5'-TCGTCG-3' moiety

[0399] The synthesis, deprotection, workup, and analysis were performed as described in Example 2.

*Example 9: Synthesis of a Chimeric Compound with a Linear Structure and Hexaethylene Glycol and Triethylene Glycol Spacers*

[0400] C-30, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, the spacer moieties and 5'-end group are hexaethylene glycol (HEG), connected to the nucleic acid moieties via phosphorothioate linkages, and the 3'-end group is triethylene glycol (TEG), connected to the nucleic acid moiety via a phosphorothioate linkage.

[0401] C-30: HEG-5'-TCGTCG-3'-HEG-5'-ACGTTCG-3'-HEG-5'-AGATGAT-3'-TEG

[0402] The C-30 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The triethylene glycol-controlled-pore glass, used as the solid support for the synthesis, was from Glen Research (Sterling, VA). The nucleoside monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-hexaethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphor amidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The HEG spacer precursor was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and HEG spacers in the

desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a triethylene glycol solid support
2. Synthesis of 5'-AGATGAT-3' moiety
3. Addition of HEG spacer
4. Synthesis of 5'-ACGTTCG-3' moiety
5. Addition of HEG spacer
6. Synthesis of 5'-TCGTCG-3' moiety
7. Addition of the HEG spacer

[0403] The synthesis, deprotection, workup, and analysis were performed as described in Example 2.

*Example 10: Synthesis of a Chimeric Compound with a Linear Structure and Hexaethylene Glycol and Triethylene Glycol Spacers, and with Phosphodiester Linkages*

[0404] C-31, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphodiester linkages, the spacer moieties and 5'-end group are hexaethylene glycol (HEG), connected to the nucleic acid moieties via phosphodiester linkages, and the 3'-end group is triethylene glycol (TEG); connected to the nucleic acid moiety via a phosphodiester linkage.

[0405] C-31: HEG-5'-TCGTCG-3'-HEG-5'-ACGTTCG-3'-HEG-5'-AGATGAT-3'-TEG

[0406] The C-31 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1 umol phosphodiester DNA. The triethylene glycol-controlled-pore glass, used as the solid support for the synthesis, was from Glen Research (Sterling, VA). The nucleoside monomers and the spacer moiety,

4,4'-O-dimethoxytrityl-hexaethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphor amidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The HEG spacer was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and HEG spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a triethylene glycol solid support
2. Synthesis of 5'-AGATGAT-3' moiety
3. Addition of HEG spacer
4. Synthesis of 5'-ACGTTCG-3' moiety
5. Addition of HEG spacer
6. Synthesis of 5'-TCGTCG-3' moiety
7. Addition of the HEG spacer

[0407] The synthesis cycle consisted of a detritylation step, a coupling step (phosphoramidite monomer plus 1H-tetrazole), a capping step, an oxidation step, and a final capping step. At the completion of assembly, the 'trityl-off' compound was cleaved from the controlled-pore glass and the bases were deprotected with concentrated aqueous ammonia at 58°C for 16 hours. The compound was purified by preparative polyacrylamide electrophoresis, desalted on a Sep-pak Plus cartridge (Waters, Milford, MA), and precipitated from 1 M aqueous sodium chloride with 2.5 volumes of 95% ethanol. The compound was dissolved in Milli Q water and the yield was determined from the absorbance at 260 nm. Finally, the compound was lyophilized to a powder. The compound was characterized by capillary gel electrophoresis, electrospray mass spectrometry, and RP-HPLC to confirm composition and purity. An endotoxin content assay (LAL assay, Bio Whittaker) was also conducted, showing endotoxin levels were <5 EU/mg compound.



*Example 11: Synthesis of a Chimeric Compound with a Linear Structure and 2-(Hydroxymethyl)ethyl Spacers*

[0408] C-25, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are 2-(hydroxymethyl)ethyl (HME); connected to the nucleic acid moieties via phosphorothioate linkages.

[0409] C-25: 5'-TCGTCG-3'-HME-5'-ACGTTCG-3'-HME-5'-AGATGAT-3'

[0410] The C-25 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1 umol phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursor, 1-O-(4,4'-dimethoxytrityl)-3-O-levulinyl-glycerol-2-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from ChemGenes, Ashland, MA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The HME spacer was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and HME spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction:

1. Use a 3'-support bound "T" solid support
2. Synthesis of 5'-AGATGA-3' moiety
3. Addition of HME spacer
4. Synthesis of 5'-ACGTTCG-3' moiety
5. Addition of HME spacer
6. Synthesis of 5'-TCGTCG-3' moiety

[0411] The synthesis, deprotection, workup, and analysis were performed as described in Example 2. The levulinyl group is removed during the treatment with ammonia.

*Example 12: Synthesis of a Chimeric Compound with a Linear Structure and a Negatively Charged Spacer Moiety*

[0412] C-13, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moiety is a propyl (C3) polymer linked via phosphorothioate linkages.

[0413] C-13: 5'-TCGTCG-3'-(C3)<sub>15</sub>-5'-T-3'

[0414] The C-13 molecule was synthesized on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturers protocol for 1 umol phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-propyl-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.1 M. The C3 spacer was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and C3 spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "T" solid support
2. Addition of 15 C3 spacers
3. Synthesis of 5'-TCGTCG-3' moiety

[0415] The synthesis cycle consisted of a detritylation step, a coupling step (phosphoramidite monomer plus 1H-tetrazole), a capping step, a sulfurization step using 0.02 M 3-amino-1,2,4-dithiazole-5-thione (ADTT) in 9:1 acetonitrile:pyridine, and a final capping step. At the completion of assembly, the 'trityl-on' compound was cleaved from the controlled-pore glass and the bases were deprotected with concentrated aqueous ammonia at 58°C for 16 hours. The compound was purified by HPLC on a Hamilton PRP-1 column using an increasing gradient of acetonitrile in 0.1 M triethylammonium acetate. The purified compound was concentrated to dryness, the 4,4'-dimethoxytrityl group was removed with 80% aqueous acetic acid, and then the compound was precipitated two times from 1 M aqueous sodium

chloride with 2.5 volumes of 95% ethanol. The compound was dissolved in Milli Q water and the yield was determined from the absorbance at 260 nm. Finally, the compound was lyophilized to a powder.

[0416] The compound was characterized by capillary gel electrophoresis, electrospray mass spectrometry, and RP-HPLC to confirm composition and purity. An endotoxin content assay (LAL assay, Bio Whittaker) was also conducted, showing endotoxin levels were <5 EU/mg compound.

[0417] C-14, C-15 and C-16 were synthesized analogously.

*Example 13: Synthesis of a Chimeric Compound with a Linear Structure and a Negatively Charged Spacer Moiety*

[0418] C-38, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are hexaethylene glycol (HEG), connected via phosphorothioate linkages.

[0419] C-38: 5'-TCGTCGA-3'-(HEG)<sub>4</sub>-5'-TCGTCGA-3'

[0420] The C-38 molecule was synthesized as described in Example 2. The spacer moiety precursor is 4,4'-O-dimethoxytrityl-hexaethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA). The synthesis was accomplished by carrying out the following steps:

1. Use a 3'-support bound "A" solid support
2. Synthesis of 5'-TCGTCG-3' moiety
3. Addition of 4 HEG spacers
4. Synthesis of 5'-TCGTCGA-3' moiety

[0421] The compound was purified using HPLC as described in Example 12. The compound was characterized and the endotoxin content determined as described in Example 2.

*Example 14: Synthesis of a Chimeric Compound with a Linear Structure and a Negatively Charged Spacer Moiety with Both Nucleic Acid Moieties Attached Via the 3'-End*

[0422] C-37, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages, and the spacer moieties are hexaethylene glycol (HEG), connected via phosphorothioate linkages.

[0423] C-37: 5'-TCGTCGA-3'-(HEG)-3'-AGCTGCT-5'

[0424] The C-37 molecule was synthesized as described in Example 2, except that a 5'-support bound nucleoside and 3'-O-(4,4'-dimethoxytrityl)-protected nucleoside-5'-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidites were used (Glen Research, Sterling, VA) to synthesize the first nucleic acid moiety. The spacer moiety precursor is 4,4'-O-dimethoxytrityl-hexaethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphor amidite (obtained from Glen Research, Sterling, VA). The synthesis was accomplished by carrying out the following steps:

1. Use a 5'-support bound "T" solid support
2. Synthesis of 3'-AGCTGC-5' moiety with 3'-O-(4,4'-dimethoxytrityl)-protected nucleoside-5'-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidites (5' to 3' synthesis)
3. Addition of 4 HEG spacers
4. Synthesis of 5'-TCGTCGA-3' moiety with 5'-O-(4,4'-dimethoxytrityl)-protected nucleoside-3'-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidites (3' to 5' synthesis)

[0425] The compound was purified using HPLC as described in Example 12. The compound was characterized and the endotoxin content determined as described in Example 2.

*Example 15: Synthesis of a Chimeric Compound with a Branched Structure*

[0426] C-27, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages and the spacer moiety is glycerol, connected to the nucleic acid moieties via phosphorothioate linkages.

[0427] C-27: (5'-TCGTCGA-3')<sub>2</sub>-glycerol-5'-AACGTTC-3'

[0428] The C-27 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursor, 1,3-di-(4,4'-O-dimethoxytrityl)-glycerol-2-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (symmetrical branched phosphoramidite obtained from ChemGenes, Ashland, MA, Figure 2) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The glycerol spacer was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and the glycerol spacer in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "C" solid support
2. Synthesis of 5'-AACGTT-3' moiety
3. Addition of the symmetrical branched phosphoramidite based on glycerol
4. Synthesis of two 5'-TCGTCGA-3' moieties simultaneously

[0429] The preparation of this branched compound followed the same protocol described in Example 2, except that in step 4, each reagent delivery in the synthesis cycle was doubled because two nucleic acid chains were built simultaneously. The symmetrical branched phosphoramidite shown in Figure 2 requires the nucleic acid sequences synthesized after the addition of the symmetrical branched phosphoramidite to be the same, although the nucleic acid sequence synthesized before its addition may be the same or different from the later sequences.

[0430] The branched compound was purified and characterized as described in Example 2.

[0431] C-28 was synthesized analogously.

*Example 16: Synthesis of a Chimeric Compound with a Branched Structure and with All Nucleic Acid Moieties Attached via the 3'-end*

[0432] C-95, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages and the spacer moieties are glycerol and HEG, connected to the nucleic acid moieties via phosphorothioate linkages.

[0433] C-95: (5'-TCGTCGA-3'-HEG)<sub>2</sub>-glycerol-HEG-3'-AGCTGCT-5'

[0434] The C-95 molecule was synthesized as described in Example 2, except that a 5'-support bound nucleoside and 3'-O-(4,4'-dimethoxytrityl)-protected nucleoside-5'-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidites were used (Glen Research, Sterling, VA) to synthesize the first nucleic acid moiety. The branched spacer moiety precursor is 1,3-di-(4,4'-O-dimethoxytrityl)-glycerol-2-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (symmetrical branched phosphoramidite obtained from ChemGenes, Ashland, MA, Figure 2). The synthesis was accomplished by carrying out the following steps:

1. Use a 5'-support bound "T" solid support
2. Synthesis of 3'-AGCTGC-5' moiety with 3'-O-(4,4'-dimethoxytrityl)-protected nucleoside-5'-O-(N,N-diisopropyl) 2-cyanoethyl phosphoramidites (5' to 3' synthesis)
3. Addition of a HEG spacer
4. Addition of the symmetrical branched phosphoramidite based on glycerol
5. Addition of two HEG spacers simultaneously

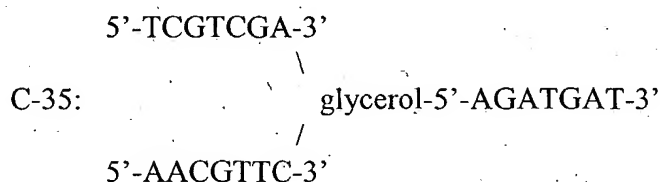
6. Synthesis of two 5'-TCGTCGA-3' moieties simultaneously with 5'-O-(4,4'-dimethoxytrityl)-protected nucleoside-3'-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidites (3' to 5' synthesis)

[0435] The preparation of this branched compound followed the same protocol described in Example 2, except that in steps 5 and 6, each reagent delivery in the synthesis cycle was doubled because two nucleic acid chains were built simultaneously. The symmetrical branched phosphoramidite shown in Figure 2 requires the nucleic acid sequences synthesized after the addition of the symmetrical branched phosphoramidite to be the same, although the nucleic acid sequence synthesized before its addition may be the same or different from the later sequences.

[0436] The compound was purified using HPLC as described in Example 12. The compound was characterized and the endotoxin content determined as described in Example 2.

*Example 17: Synthesis of a Chimeric Compound with a Branched Structure, Containing Three Different Nucleic Acid Moieties*

[0437] C-35, having the formula shown below, is synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages and the spacer moiety is glycerol, connected to the nucleic acid moieties via phosphorothioate linkages.



[0438] The C-35 molecule is synthesized as described in Example 2. The nucleoside monomers and the spacer moiety precursor, 1-(4,4'-O-dimethoxytrityl)-3-O-levulinyl-glycerol-2-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (asymmetrical branched phosphoramidite obtained from ChemGenes, Ashland, MA,

Figure 2) are dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The glycerol spacer is placed in an auxiliary monomer site on the instrument. The instrument is programmed to add the nucleotide monomers and the glycerol spacer in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "T" solid support
2. Synthesis of 5'-AGATGA-3' moiety
3. Addition of the asymmetrical branched phosphoramidite based on glycerol
4. Synthesis of the 5'-AACGTTC-3' moiety at the dimethoxytrityl end
5. Detritylation and capping of the AACGTTC moiety
6. Removal of the levulinyl protecting group
7. Synthesis of the 5'-TCGTCGA-3' moiety

[0439] Synthesis takes place essentially as described in Example 2, except that after step 4, the 5'-AACGTTC-3' moiety is detritylated and capped with acetic anhydride/N-methylimidazole in order to terminate that nucleic acid moiety. Next, the levulinyl protecting group is removed with 0.5 M hydrazine hydrate in 3:2 pyridine:acetic acid/pH 5.1 for 5 min. The compound-containing solid support is washed well with anhydrous acetonitrile, and the 5'-TCGTCGA-3' moiety is added using the protocol described in Example 2.

[0440] The branched compound is purified and characterized as described in Example 2.

*Example 18: Synthesis of a Chimeric Compound with a Branched Structure by a Conjugation Strategy*

[0441] C-36 is synthesized as shown in Figure 3. The nucleic acid moieties are DNA with phosphorothioate linkages and the spacer moiety is based on a



STARBURST® dendrimer. The nucleic acid moiety is synthesized with a 5'-C6-disulfide spacer (thiol-modifier C6 S-S, Glen Research, Sterling, VA product no. 10-1926-xx), which upon reduction, provides a thiol group that can react with the maleimide groups on the dendrimer.

Synthesis of 5'-C6-disulfide-TCGTCGA (4):

[0442] The 5'-C6-disulfide-TCGTCGA is synthesized using a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The nucleoside monomers and the thiol-modifier C6 S-S (Glen Research, Sterling, VA) are dissolved in anhydrous acetonitrile to a final concentration of 0.1 M. The thio-modifier is placed in an auxiliary monomer site on the instrument. The instrument is programmed to add the nucleotide monomers and the thiol modifier in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "A" solid support
2. Synthesis of 5'-TCGTCG-3' moiety
3. Addition of the thiol modifier precursor (S-trityl-6-mercaptohexyl)-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite)

[0443] The synthesis cycle consists of a detritylation step, a coupling step (phosphoramidite monomer plus 1H-tetrazole), a capping step, a sulfurization step using 0.02 M 3-amino-1,2,4-dithiazole-5-thione (ADTT) in 9:1 acetonitrile:pyridine, and a final capping step. At the completion of assembly, the 'trityl-on' compound is cleaved from the controlled-pore glass and the bases are deprotected with concentrated aqueous ammonia at 58°C for 16 hours. The compound is purified by HPLC on a Hamilton PRP-1 column using an increasing gradient of acetonitrile in 0.1 M triethylammonium acetate. The purified compound is concentrated to dryness, the 4,4'-dimethoxytrityl group is removed with 80% aqueous acetic acid, and then the

compound is precipitated two times from 1 M aqueous sodium chloride with 2.5 volumes of 95% ethanol. The compound is dissolved in Milli Q water and the yield is determined from the absorbance at 260 nm. Finally, the compound is lyophilized to a powder.

[0444] The compound is characterized by capillary gel electrophoresis, electrospray mass spectrometry, and RP-HPLC to confirm composition and purity. An endotoxin content assay (LAL assay, Bio Whittaker) is also conducted, showing endotoxin levels were <5 EU/mg compound.

Synthesis of 5'-thiol-C6-TCGTCGA (5):

[0445] The disulfide modified nucleic acid (4) is reduced to a thiol using tris(2-carboxyethylphosphine) hydrochloride (TCEP; Pierce, Rockford, IL). The nucleic acid is dissolved at a concentration of 20 mg/ml in buffer containing 0.1 M sodium phosphate/0.15 M sodium chloride/pH 7.5. In a separate vial, the TCEP is dissolved to a concentration of 0.17 M in 0.1 M sodium phosphate/0.15 M sodium chloride/pH 7.5. Add 5 equivalents of TCEP to the nucleic acid and mix gently. Incubate the solution for 120 min at 40°C and then purify by size exclusion chromatography (Pharmacia P2 column) to yield the 5'-thiol-C6-TCGTCGA (5).

Synthesis of the maleimide-modified STARBURST® dendrimer (7):

[0446] STARBURST® dendrimers with various numbers of amines (4, 8, 16, 32, 64, etc.) are available from Aldrich (Milwaukee, WI). A Starburst® dendrimer (6), having four amino groups, is dissolved in dimethylformamide (DMF) at a concentration of 0.2 M. Triethylamine (10 equivalents) and sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC; Pierce, Rockford, IL, 8 equivalents) are then added and the solution is stirred for 2 hours or until complete, as determined by thin layer chromatography (TLC; 10% methanol/dichloromethane). The reaction is quenched with water for 30 min and then the DMF is removed *in*

*vacuo*. The residue is dissolved in dichloromethane and washed two times with aqueous saturated sodium bicarbonate and then water. The organic phase is dried over  $\text{MgSO}_4$ , filtered, and concentrated to dryness *in vacuo*. The product is purified by silica gel chromatography to yield 7.

Synthesis of STARBURST® dendrimer-(5'-TCGTCGA-3')<sub>4</sub> (8):

[0447] The maleimide-modified STARBURST® dendrimer (6) is dissolved in DMSO (5 mg/ml) and the purified 5'-C6-thiol-TCGTCGA (5) (10 equivalents), dissolved at a concentration of 10 mg/ml in 0.1 M sodium phosphate/ 0.15 M sodium chloride/pH 7.5, is added drop-wise. The resulting mixture is stirred at 40°C overnight. The conjugate is purified by size exclusion chromatography (Sephadex G-25) to yield compound 8.

*Example 19: Synthesis of a Chimeric Compound with a Branched Structure*

[0448] C-94, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages and the spacer moiety is glycerol; connected to the nucleic acid moieties via phosphorothioate linkages.

[0449] C-94: (5'-TCGTCGA-3'-HEG)<sub>2</sub>-glycerol-HEG-5'-TCGTCGA-3'

[0450] The C-94 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturers protocol for 1  $\mu\text{mol}$  phosphorothioate DNA. The nucleoside monomers and the spacer moiety precursors [1,3-di-(4,4'-O-dimethoxytrityl)-glycerol-2-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (symmetrical branched phosphoramidite obtained from ChemGenes, Ashland, MA, Figure 2) and 4,4'-O-dimethoxytrityl-hexaethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA)] were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The glycerol and HEG spacers were placed in auxiliary monomer sites on the instrument. The instrument was programmed to add the nucleotide monomers, HEG spacers, and the

glycerol spacer in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "A" solid support
2. Synthesis of 5'-TCGTCGA-3'-moiety
3. Addition of HEG spacer
4. Addition of the symmetrical branched phosphoramidite based on glycerol
5. Addition of two HEG spacers simultaneously
6. Synthesis of two 5'-TCGTCGA-3' moieties simultaneously

**[0451]** The preparation of this branched compound followed the same protocol described in Example 2, except that in steps 5 and 6, each reagent delivery in the synthesis cycle was doubled because two nucleic acid chains were built simultaneously. The symmetrical branched phosphoramidite shown in Figure 2. requires the nucleic acid sequences synthesized after the addition of the symmetrical branched phosphoramidite to be the same, although the nucleic acid sequence synthesized before its addition may be the same or different from the later sequences.

**[0452]** The branched compound was purified by HPLC as described in Example 12 and characterized as described in Example 2.

**[0453]** C-96 and C-101 were synthesized analogously.

**[0454]** C-103 and C-104 were also synthesized by the same method, except that either triethylene glycol or propyl spacers were used, respectively, in place of the hexaethylene glycol spacers.

*Example 20: Synthesis of a Chimeric Compound with a Branched Structure*

**[0455]** C-98, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with phosphorothioate linkages and the spacer moiety is glycerol, connected to the nucleic acid moieties via phosphorothioate linkages.

**[0456]** C-98: (5'-TCGTCGA-3'-HEG)<sub>3</sub>-trebler-HEG-5'-AACGTTC-3'-HEG-5'-TCGA-3'

**[0457]** The C-98 molecule was synthesized by TriLink BioTechnologies (SanDiego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturers protocol for 1 umol phosphorothioate DNA. The nucleoside monomers and the spacer moieties [trebler phosphoramidite (obtained from Glen Research, Sterling, VA) and 4,4'-O-dimethoxytrityl-hexaethylene glycol-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen Research, Sterling, VA)] were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The trebler and HEG spacers were placed in auxiliary monomer sites on the instrument. The instrument was programmed to add the nucleotide monomers, HEG spacer and the trebler spacer in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "A" solid support
2. Synthesis of 5'-TCGA-3'-moiety
3. Addition of HEG spacer
4. Synthesis of the 5'-AACGTTC-3' moiety
5. Addition of HEG spacer
6. Addition of the trebler phosphoramidite (see Fig. 2)
7. Addition of three HEG spacers simultaneously
8. Synthesis of three 5'-TCGTCGA-3'-moieties simultaneously

**[0458]** The preparation of this branched compound followed the same protocol described in Example 2, except that in steps 7 and 8, each reagent delivery in the synthesis cycle was tripled because 3 nucleic acid chains were built simultaneously. The symmetrical treblerphosphoramidite shown in Figure 2 requires the nucleic acid sequences synthesized after the addition of the symmetrical treblerphosphoramidite to be the same, although the nucleic acid sequence synthesized before its addition may be the same or different from the later sequences.

[0459] The branched compound was purified by HPLC as described in Example 12, and characterized as described in Example 2.

*Example 21: Synthesis of a Linear Chimeric Compound with Hexaethylene Glycol Spacers and a 3'-Thiol Linker*

[0460] CICs containing 3'-thiol linkers are first synthesized and purified as their disulfide derivatives. The disulfide group is then reduced to yield the reactive thiol group. For example, to synthesize C-116, C-8 was synthesized as in Example 2, except that 3'-Thiol Modifier C3 S-S CPG (Glen Research, Sterling, VA) was used as the solid support instead of the "T" solid support.

[0461] C-116: 5'-TCGTCGA-3'-HEG-5'-ACGTTCG-3'-HEG-5'-AGATGAT-3'-(CH<sub>2</sub>)<sub>3</sub>SS(CH<sub>2</sub>)<sub>3</sub>OH

[0462] It will be appreciated that C-116 can be described as [C-8]-3'-disulfide. The CIC was purified by HPLC as described in Example 12. The compound was characterized as described in Example 2.

[0463] C-116 was reduced to the thiol using tris(2-carboxyethylphosphine) hydrochloride (TCEP; Pierce, Rockford, IL). C-116 was dissolved to a concentration of 30.5 mg/ml (0.8 ml, 24.4 mg; 3.14  $\mu$ mol) in 100 mM sodium phosphate/150 mM sodium chloride/1 mM EDTA/pH 7.4 buffer. In a separate vial, TCEP was dissolved to a concentration of 0.167 M in 100 mM sodium phosphate/150 mM sodium chloride/1 mM EDTA/pH 7.4 buffer. 5 equivalents (100  $\mu$ l, 4.8 mg, 17  $\mu$ mol) of the TCEP stock solution were added to the CIC solution. The solution was mixed gently, incubated for 120 min at 40°C, and purified on a Sephadex G-25 column (5 ml, Amersham Pharmacia, Piscataway, NJ) to yield C-117 (13.2 mg). It will be appreciated that C-117 can be described as [C-8]-3'-thio. The CIC was purified by HPLC as described in Example 12.

[0464] C-115 was synthesized analogously from C-114.

*Example 22: Synthesis of a Linear Chimeric Compound with Propyl Spacers and a 5'-Thiol Linker*

[0465] CICs containing 5'-thiol linkers are first synthesized and purified as their disulfide derivatives. The disulfide group is then reduced to yield the reactive thiol group. Compound C-110 (below) can be described as 5'-disulfide-C-11. Compound C-111 can be described as 5'-thiol-C-11.

[0466] C-110: HO(CH<sub>2</sub>)<sub>6</sub>SS(CH<sub>2</sub>)<sub>6</sub>-5'-TCGTCG-3'-C3-5'-ACGTTTCG-3'-C3-5'-AGATGAT-3'

[0467] C-110 was synthesized as described in Example 3, except that the final coupling was with the thiol modifier C6 S-S (Glen Research, Sterling, VA). The CIC was purified by HPLC as described in Example 12. The compound was characterized as described in Example 2. C-110 was reduced to the thiol using tris(2-carboxyethylphosphine) hydrochloride (TCEP; Pierce, Rockford, IL) as described in Example 22.

[0468] C-107, C-113 and P-16 were synthesized analogously.

*Example 23: Synthesis of a Chimeric Compound with a Branched Structure by a Conjugation Strategy*

[0469] C-105 was synthesized as shown in Figure 4. Tris(2-maleimidoethyl)amine (TMEA, Pierce, Rockford, IL) was dissolved to a concentration of 4.3 mg/ml in dimethylformamide (DMF). The TMEA solution (12 ul, 52 ug, 1.0 eq) was added to a solution of C-117 (237 ul, 4.0 mg, 4.0 eq) in 100 mM sodium phosphate/150 mM sodium chloride/1 mM EDTA/pH 7.4 buffer and mixed well. The solution was left at room temperature overnight and was purified on a Superdex 200 column (24 ml, Amersham Pharmacia, Piscataway, NJ) in 10 mM sodium phosphate/141 mM sodium chloride/pH 7.0 buffer. The product was dried *in vacuo*, dissolved in 0.4 ml of Milli Q water, and precipitated with 1.0 ml of 95% ethanol. After freezing at -20°C for 1 hour, the mixture was centrifuged (2 min at 14

K RPM), and the supernatant was carefully removed. The pellet was dissolved in 0.35 ml of Milli Q water and the concentration of C-105 was measured (0.4 mg isolated). The compound was analyzed as described in Example 2.

[0470] C-99 was synthesized analogously.

*Example 24: Synthesis of a Chimeric Compound with a Branched Structure by a Conjugation Strategy*

A. Synthesis of Maleimido-STARBURST DENDRIMER® Generation 2

[0471] The STARBURST dendrimer®, Generation 2, containing 16 hydroxyl groups, was purchased as a 20% solution in methanol from Aldrich (Milwaukee, WI). The dendrimer (191 ul, 38.2 mg, 11.7 umol) was dried *in vacuo*, re-dissolved in 200 ul of DMF and re-dried *in vacuo* to remove the last traces of methanol. To prepare the maleimido-dendrimer, N-(p-maleimidophenyl)isocyanate (PMPI, 50 mg, 233.5 umol) was dissolved in 200 ul of DMF in a separate glass vial and then quickly added to the dendrimer. The mixture was vortexed until the dendrimer dissolved. The solution was put on a rotating mixer overnight at room temperature. The solution was concentrated *in vacuo*, dissolved in 20% methanol/dichloromethane (1 ml), and purified on a 7.5 g silica gel column (70-230 mesh, 60 Å) in 20% methanol/dichloromethane. The maleimido-dendrimer product eluted from the column in the first fraction (due to the presence of residual DMF) and was free of the PMPI by-products. The product was concentrated to a tan solid (10 mg, 13% yield).

B. Synthesis of STARBURST DENDRIMER®-(5'-TGACTGTGAACGT TCGAGATGA)<sub>x=3-16</sub> (SEQ ID NO:2) (C-102)

[0472] The maleimido-dendrimer (5.7 mg) was dissolved in dimethylsulfoxide (DMSO) to form a stock solution at a concentration of 2.5 mg/ml. The maleimido-dendrimer stock solution (100 ul, 0.25 mg, 0.0375 umol) was added to a solution of C-107 (9.1 mg, 1.2 umol) in 100 mM sodium phosphate/150 mM sodium chloride/1



mM EDTA/pH 7.4 buffer (0.7 ml). The solution was placed on a rotating mixer overnight at room temperature and the product was purified on a Superdex 200 column (24 ml, Amersham Pharmacia, Piscataway, NJ) in 10 mM sodium phosphate/141 mM sodium chloride/pH 7.0 buffer. The product eluted in the void volume at 10.4 min (1.3 mg). The product was found to be a mixture of high molecular weight species, representing different loadings of polynucleotide on the dendrimer, by analysis on a 1.2% agarose E-gel (Invitrogen, Carlsbad, CA). C-102 ran as a mixture of products between 1 kb to greater than 15 kb (effective size compared to double-stranded DNA markers).

*Example 25: Synthesis of a Linear Chimeric Compound with Propyl Spacers and Mixed Phosphodiester/Phosphorothioate Linkages*

**[0473]** C-84, having the structure shown below, was synthesized. The nucleic acid moieties are DNA with either phosphorothioate linkages, indicated by a lower case "s", or phosphodiester linkages (all other linkages), and the spacer moieties are propyl (C3), connected to the nucleic acid moieties via phosphodiester linkages.

**[0474]** C-84: 5'-GsGs-3'-C3-5'-TGC-3'-C3-5'-ATCGAT-3'-C3-5'-GCA-3'-C3-5'-GGsGsGsGsG-3'

(where a lower case "s" indicates a phosphorothioate linkage and the other linkages are phosphodiester)

**[0475]** The C-84 molecule was synthesized by TriLink BioTechnologies (San Diego, CA) on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer using the manufacturer's protocol for 1  $\mu$ mol phosphorothioate DNA. The phosphorothioate linkages were programmed using upper case letters for the bases and the phosphodiester linkages were programmed using lower case letters for the bases and auxiliary positions containing the propyl spacer phosphoramidite. The nucleoside monomers and the spacer moiety precursor, 4,4'-O-dimethoxytrityl-propyl-O-(N,N-diisopropyl) 2-cyanoethylphosphoramidite (obtained from Glen

Research, Sterling, VA) were dissolved in anhydrous acetonitrile to a final concentration of 0.05 M. The C3 spacer was placed in an auxiliary monomer site on the instrument. The instrument was programmed to add the nucleotide monomers and C3 spacers in the desired order, with synthesis of the nucleic acid moieties occurring in the 3' to 5' direction.

1. Use a 3'-support bound "G" solid support
2. Synthesis of 5'-GGsGsGsGsG-3'
3. Addition of C3 spacer
4. Synthesis of 5'-GCA-3'
5. Addition of C3 spacer
6. Synthesis of 5'-ATCGAT-3'
7. Addition of C3 spacer
8. Synthesis of 5'-TGC-3'
9. Addition of C3 spacer
10. Synthesis of 5'-GsGs-3'

[0476] The synthesis, deprotection, workup, and analysis were performed as described in Example 2.

[0477] C-85 and C-87 were synthesized analogously.

*Example 26: Synthesis of Oligonucleotides Containing Fewer Than Eight (8)*

*Nucleotides*

[0478] Polynucleotides containing fewer than eight bases and containing phosphorothioate linkages were synthesized on a Perseptive Biosystems Expedite 8909 automated DNA synthesizer. Polynucleotides were purified by RP-HPLC on a Polymer Labs PLRP-S column using an increasing gradient of acetonitrile in 0.1 M triethylammonium acetate. The purified polynucleotides were concentrated to dryness, the 4,4'-dimethoxytrityl group was removed with 80% aqueous acetic acid, and then the compounds were precipitated two times from 0.6 M aqueous sodium

acetate/pH 5.0 with 3 volumes of isopropanol. The polynucleotides were dissolved in Milli Q water and the yield was determined from the absorbance at 260 nm. Finally, the polynucleotides were lyophilized to a powder. The polynucleotides were characterized, and the endotoxin content determined, as described in Example 2.

*Example 27: Preparation of Cationic Biodegradable Microcarriers*

[0479] Cationic poly(lactic acid, glycolic acid) microcarriers (cPLGA) were prepared as follows. 0.875 g of poly (D,L-lactide-co-glycolide) 50:50 polymer (Boehringer Mannheim, Indianapolis, IN) with an intrinsic viscosity of 0.41 dl/g (0.1%, chloroform, 25°C) was dissolved in 7.875 g of methylene chloride at 10% w/w concentration, along with 0.3 g of DOTAP. The clear organic phase was then emulsified into 500 ml of PVA aqueous solution (0.35% w/v) by homogenization at 4000 rpm for 30 minutes at room temperature using a laboratory mixer (Silverson L4R, Silverson Instruments). System temperature was then raised to 40°C by circulating hot water through the jacket of the mixing vessel. Simultaneously, the stirring rate was reduced to 1500 rpm, and these conditions were maintained for 2 hours to extract and evaporate methylene chloride. The microsphere suspension was allowed to cool down to room temperature with the help of circulating cold water.

[0480] Microcarriers were separated by centrifugation at 8000 rpm for 10 minutes at room temperature (Beckman Instruments) and resuspended in deionized water by gentle bath sonication. The centrifugal wash was repeated two additional times to remove excess PVA from the particle surface. Final centrifugal pellets of particles were suspended in approximately 10 ml of water, and lyophilized overnight. The dried cationic microcarrier powder was characterized for size and surface charge: mean size (number weighted,  $\mu$ ) = 1.4; zeta potential (mV) = 32.4.

*Example 28: Immunomodulation of Human Cells by CICs*

[0481] Tests were conducted to assess the immunomodulatory activity of (1) chimeric molecules containing spacer moieties and (2) polynucleotides.

[0482] The chimeric compounds and polynucleotides were synthesized as described *supra* or by conventional phosphorothioate chemistry. Polynucleotides P-6 and P-7 were synthesized by Hybridon Specialty Products (Milford MA). Immunomodulatory activity was determined by routine assays as disclosed herein.

[0483] Peripheral blood was collected from volunteers by venipuncture using heparinized syringes. Blood was layered onto a FICOLL® (Amersham Pharmacia Biotech) cushion and centrifuged. PBMCs, located at the FICOLL® interface, were collected, then washed twice with cold phosphate buffered saline (PBS). The cells were resuspended and cultured in 48 well plates (Examples 29-32) or 96-well plates (Examples 33-40) at  $2 \times 10^6$  cells/mL at 37°C in RPMI 1640 with 10% heat-inactivated human AB serum plus 50 units/mL penicillin, 50 µg/mL streptomycin, 300 µg/mL glutamine, 1 mM sodium pyruvate, and 1 x MEM non-essential amino acids (NEAA).

[0484] The cells were cultured in the absence of test samples, in the presence of test samples at 20 µg/ml (0.5 OD/ml), or in the presence of test samples at 20 µg/ml premixed with 100 µg/ml cPLGA (when used) for 24 hours. Cell-free medium was then collected from each well and assayed for IFN-γ and IFN-α concentrations. SAC (Pansorbin CalBiochem, 1/5000 dilution) was used as a positive control. SAC contains is *Staph. aureus* (cowan) cell material.

[0485] IFN-γ and IFN-α were assayed using CYTOSCREEN™ ELISA kits from BioSource International, Inc., according to the manufacturer's instructions.

[0486] In the human PBMC assay, background levels of IFN-γ can vary, even significantly, with the donor. Levels of IFN-α generally exhibit low background levels under unstimulated conditions.

[0487] Examples of results from such assays are shown in Examples 29-40 below.

[0488] In each of the experiments shown, "medium alone" and "P-7" are negative controls. "P-7" has been previously shown not to have immunostimulatory activity. SAC and "P-6" are positive controls. P-6 has been previously shown to have significant immunostimulatory activity.

*Example 29 Immunostimulatory Activity of CICs*

[0489] This example shows that four different CICs had significant immunomodulatory activity as evidenced by stimulation of IFN- $\gamma$  and IFN- $\alpha$  secretion (Table 3). As expected, P-7 had no activity. In addition, P-1, a TCG-containing 7-mer, had no activity. Interestingly, CICs with HEG and propyl spacer moieties showed different degrees of stimulation of IFN- $\alpha$  secretion. Although both types of CICs stimulated IFN- $\alpha$  secretion, the effect was more marked for the HEG-containing CICs.

Table 3

Test compound	IFN- $\gamma$ (pg/ml)			IFN- $\alpha$ (pg/ml)		
	Donor 1	Donor 2	mean	Donor 1	Donor 2	mean
medium alone	8	0	4	0	0	0
P-7	410	51	231	0	0	0
SAC	2040	1136	1588	393	43	218
P-6	2180	669	1425	401	39	220
P-1	8	0	4	0	0	0
C-8	1916	696	1306	1609	44	827
C-9	2157	171	1164	142	0	71
C-10	1595	952	1273	1662	50	856
C-11	2308	270	1289	119	0	59

*Example 30 Activity of Polynucleotides*

**[0490]** This example shows that polynucleotides P-1, P-2, P-3, P-4 and P-5 did not have immunomodulatory activity (Table 4). These polynucleotides have the sequences of the nucleic acid moieties of C-10 and C-11, shown in Example 29 to have immunomodulatory activity.

Table 4

Test compound	IFN- $\gamma$ (pg/ml)			IFN- $\alpha$ (pg/ml)		
	Donor 3	Donor 4	mean	Donor 3	Donor 4	mean
medium alone	0	3	2	0	18	9
P-7	3	8	5	0	31	15
SAC	1179	2000	1589	50	969	510
P-6	99	223	161	28	106	67
P-1	1	4	2	0	32	16
P-3	1	3	2	0	32	16
P-4	0	3	1	0	58	29
P-5	0	3	2	0	57	29
P-2	0	4	2	0	40	20

*Example 31 Activity of Polynucleotide Mixtures*

[0491] This example shows a mixture of polynucleotides P-1 and P-3, or P-1, P-3, P-4 and P-5 did not have immunomodulatory activity (Table 5). These polynucleotides have the sequences of the nucleic acid moieties of C-10 and C-11 which did have immunomodulatory activity. The mixtures contained equal amounts of each polynucleotide, at a total concentration of 20 µg/ml total polynucleotide.

Table 5

Test compound	IFN-γ (pg/ml)			IFN-α (pg/ml)		
	Donor 5	Donor 6	mean	Donor 5	Donor 6	mean
medium alone	3	52	28	20	20	20
P-7	7	66	37	20	94	57
SAC	903	284	593	458	8215	4337
P-6	73	1170	621	54	482	268
(P-1) + (P-3)	3	36	19	20	40	30
(P-1) + (P-3) + (P-4) + (P-5)	1	99	50	70	65	68
C-10	102	806	454	91	1700	896
C-11	25	792	409	76	175	126

*Example 32 Immunomodulatory Activity of CICs*

**[0492]** This example shows the immunomodulatory activity of C-10 and C-11, in an assay with different donors than Examples 29 and 31 (Table 6).

Table 6

Test compound	IFN- $\gamma$ (pg/ml)			IFN- $\alpha$ (pg/ml)		
	Donor 7	Donor 8	mean	Donor 7	Donor 8	mean
medium alone	1	0	1	0	0	0
P-7	2	2	2	0	0	0
SAC	594	1100	847	22	303	163
P-6	15	367	191	4	59	32
C-10	23	198	111	46	539	293
C-11	5	419	212	6	56	31

*Example 33 Immunomodulatory Activity of CICs*

**[0493]** This example shows immunomodulatory activity of C-8 and C-9, in an assay with different donors than Example 29 (Table 7). P-2, a TCG-containing 6-mer, had no activity.

Table 7

	IFN- $\gamma$					IFN- $\alpha$				
	Donor 9	Donor 10	Donor 11	Donor 12	mean	Donor 9	Donor 10	Donor 11	Donor 12	mean
medium alone	17	1	1	10	7	4	2	2	15	6
P-7	5	2	3	2	3	0	3	1	5	2
SAC	380	688	159	73	325	2246	364	1129	1029	1192
P-6	66	20	72	23	45	12	28	12	12	16
P-2	2	3	1	2	2	0	2	1	4	2
C-8	312	35	31	28	102	58	30	18	49	39
C-9	134	7	56	30	56	8	10	1	15	8



*Example 34 Immunomodulatory Activity of CICs*

**[0494]** The assays shown in Table 8 demonstrate immunostimulatory activity of several CICs of the invention, *i.e.*, CICs characterized by a variety of different short nucleic acid moieties and a variety of different spacer moieties. Table 8 also shows that compound M-1, which has a mixed HEG/nucleic acid structure but lacks any 5'-C,G-3' sequence (see Table 2), as well as certain other compounds (C-19), did not show activity. The formulation of the CICs with cPLGA significantly enhanced induction of IFN- $\alpha$ . IFN- $\gamma$  levels were also increased in some cases. The numbers "28--" represent individual donors.

TABLE 8

Conc		IFN- $\gamma$ (pg/ml)					IFN- $\alpha$ (pg/ml)				
stim	ug/ml	28065	28066	28067	28068	mean	28065	28066	28067	28068	mean
cells alone	0	96	2	1	2	25	0	4	0	6	3
P-6	20	439	12	28	906	346	14	17	45	126	50
P-7	20	397	1	8	15	105	0	8	0	3	3
P-2	20	79	1	1	0	20	0	3	0	0	1
P-3	20	94	27	1	0	31	0	0	5	0	1
P-4	20	93	1	1	1	24	0	0	9	0	2
P-2 + P-3 + P-4	20 tot; 6.7 ea	99	0	1	0	25	0	0	8	0	2
C-8	20	1000	19	56	419	373	123	6	96	358	146
C-9	20	1000	8	57	510	394	13	0	22	64	25
C-10	20	1000	9	51	559	405	116	6	107	340	142
C-17	20	1000	6	32	459	374	21	0	22	95	34
C-18	20	1000	102	27	695	456	51	9	16	162	59
C-19	20	84	8	1	2	24	0	1	0	13	4
C-20	20	354	13	16	505	222	21	5	13	64	26
C-21	20	653	16	24	960	413	227	24	183	769	300
C-23	20	438	5	6	238	172	52	3	19	137	53
C-24	20	337	2	4	116	115	28	0	8	67	26
C-25	20	541	6	19	337	226	11	0	22	79	28
M-1	20	157	1	40	2	50	0	0	3	0	1
C-27	20	475	3	24	226	182	3	0	24	16	11
C-28	20	1082	5	42	410	385	3	0	29	52	21
PLGA	0	55	1	1	5	16	0	2	12	10	6
P-6 + PLGA	20	975	191	287	573	506	388	194	565	2000	787
P-7 + PLGA	20	19	27	6	11	15	0	5	0	0	1
P-2 + PLGA	20	357	138	104	443	261	982	708	2100	2336	1532
P-3 + PLGA	20	134	1	1	4	35	307	0	0	0	77
P-4 + PLGA	20	19	1	0	3	6	34	5	0	0	10
P-2 + P-3 + P-4 + PLGA	20 tot; 6.7 ea	122	4	14	70	53	1820	0	435	106	590
C-8 + PLGA	20	527	280	245	357	352	2395	538	4380	4625	2985
C-9 + PLGA	20	334	139	343	456	318	1093	130	1686	2045	1239
C-10 + PLGA	20	619	152	557	420	437	2049	369	3515	3586	2380
C-17 + PLGA	20	508	184	587	355	408	1914	240	2729	2774	1914
C-18 + PLGA	20	732	108	355	448	411	2188	375	3513	7141	3304
C-19 + PLGA	20	1000	780	730	466	744	5997	3753	14359	7079	7797
C-20 + PLGA	20	1055	256	270	488	517	1044	191	1265	2000	1125
C-21 + PLGA	20	682	874	390	481	607	2468	784	3372	4962	2897
C-23 + PLGA	20	216	161	120	377	219	789	189	1573	2000	1138
C-24 + PLGA	20	236	47	188	707	295	31	20	772	340	291
C-25 + PLGA	20	427	179	289	499	348	414	87	1082	1335	730
M-1 + PLGA	20	7	1	3	5	4	0	0	8	5	3
C-27 + PLGA	20	888	205	235	466	448	136	44	388	259	207
C-28 + PLGA	20	860	88	489	415	463	216	73	401	520	303
SAC	0	1000	339	511	355	551	284	156	1544	350	583

[0495] It will be apparent from review of Table 8 that Donor 28065 exhibited high background in the IFN-gamma assay. Values rendered as "1000" indicate a measurement outside the limits of sensitivity of the assay.

*Example 35 Activity of CIC Containing 3-Nucleotide Nucleic Acid Moieties and Enhancement of Activity by cPLGA*

[0496] This example shows immunostimulatory activity of several CICs in the presence and absence of cPLGA as assayed using human PBMCs. Interestingly, the phosphodiester version of C-30 (C-31) was inactive as a CIC alone, but had good activity when formulated with cPLGA. In fact, the general trend was that while the CICs containing all phosphodiester linkages (C-31, C-36, and C-93) were inactive as CICs alone, they became significantly more active when formulated with cPLGA.

[0497] C-32, a CIC containing only trimeric nucleic acid moieties, had activity when used alone and demonstrated more activity when formulated with cPLGA. See Table 9.

TABLE 9

stim	IFN- $\gamma$ (pg/ml)					IFN- $\alpha$ (pg/ml)				
	28089	28090	28098	28099	mean	28089	28090	28098	28099	mean
cells alone	0	0	0	4	1	25	79	33	28	41
P-6	84	255	745	125	302	0	62	105	105	68
P-7	0	4	0	2	1	0	27	19	37	21
C-10	35	44	174	140	98	17	61	187	304	142
C-21	61	68	218	124	118	56	157	286	466	241
C-22	31	15	110	91	62	0	46	97	247	97
C-8	62	52	205	116	109	21	124	314	362	205
C-9	12	7			10	10	67			39
C-29	63	50	150	177	110	75	92	359	332	214
C-30	0	6	12	20	9	134	29	52	47	65
C-31	0	0	0	2	0	158	26	62	29	69
C-32	0	5	11	35	13	285	31	46	59	106
C-33	0	0	0	3	1	56	22	34	30	36
C-93	0	0	0	3	1	0	30	25	29	21
C-28	14	15	183	45	64	0	64	42	67	43
PLGA	15	2	16	10	11	8	38	39	49	33
P-6 + PLGA	606	144	3277	160	1047	197	103	340	91	183
P-7 + PLGA	121	3	91	5	55	7	85	36	47	44
C-10 + PLGA	804	373	1501	301	745	523	256	509	1317	651
C-21 + PLGA	1138	454	1612	630	958	1347	1020	1001	2302	1418
C-22 + PLGA	772	244	1271	357	661	619	386	604	1339	737
C-8 + PLGA	668	332	1863	506	842	1005	683	934	1680	1075
C-9 + PLGA	1036	330			683	308	363			335
C-29 + PLGA	825	477	1536	341	795	909	711	855	1419	973
C-30 + PLGA	97	233	447	41	205	44	116	49	33	60
C-31 + PLGA	256	327	1597	406	647	696	912	1028	1361	999
C-32 + PLGA	454	192	259	57	240	281	289	218	131	230
C-33 + PLGA	171	186	249	96	176	658	1220	1764	1304	1237
C-93 + PLGA	427	628	1707	323	771	990	1738	2681	4000	2352
C-28 + PLGA	683	306	2252	224	866	136	155	141	70	126
SAC	195	489	101	306	273	67	239	92	70	117

*Example 36 Immunostimulatory Activity of CICs Containing 5' TCG.*

**[0498]** This example shows immunomodulation by CICs containing various nucleic acid moieties (see Table 10). In general, sequences containing a 5'-TCG-3' (C-8, C-21, C-50, C-51, etc.) or 5'-NTCG (C-46), where N is any nucleoside, were more active than other CG-containing CICs (C-24, C-52). Additionally, while most of the CICs induced a significant amount of IFN- $\gamma$ , the results were more variable for the induction of IFN- $\alpha$ , suggesting the motif requirements for IFN- $\alpha$  induction may be more stringent than those for IFN- $\gamma$  induction. In particular, CICs containing a 5'-TCGA-3' (C-50, C-51, C-45) generated more IFN- $\alpha$  than CICs containing a 5'-TCGT-3' (C-41, C-42, C-52).

**[0499]** With the exception of C-8 and C-21 (including the motif 5'-TCGTCGA-3'), the best IFN- $\alpha$  induction was generated by CICs with the TCGA in the 5' position.

**[0500]** CICs containing only hexameric (C-22), pentameric (C-43), and tetrameric (C-44) nucleic acid moieties were found to induce IFN- $\gamma$  when used alone. In addition, each of these CICs, as well as C-32 containing only trimeric nucleic acid moieties, induced considerable IFN- $\gamma$  and IFN- $\alpha$  when formulated with cPLGA. C-39, a CIC with two heptameric nucleic acid moieties, was active when used alone, while C-40, a CIC with one hexameric and one tetrameric nucleic acid moiety, was inactive in this experiment. Both of these CICs exhibited significant activity when formulated with cPLGA.

TABLE 10

stim	IFN- $\gamma$ (pg/ml)					IFN- $\alpha$ (pg/ml)				
	28042	28043	28044	28045	mean	28042	28043	28044	28045	mean
cells alone	15	4	3	5	7	0	44	9	0	13
P-6	495	1189	925	212	705	27	85	36	21	42
P-7	66	76	26	13	45	0	11	22	20	13
C-8	468	939	1000	234	660	20	51	32	5	27
C-24	148	156	312	26	161	0	0	8	0	2
C-21	790	1519	1198	177	921	57	72	79	15	56
C-42	198	1067	4000	37	1326	0	29	24	0	13
C-41	174	1075	841	45	534	0	3	23	0	7
C-45	590	1466	984	253	823	62	123	152	14	88
C-46	399	814	480	63	439	24	73	26	3	31
C-47	112	537	142	17	202	20	0	0	0	5
C-50	1324	1292	509	192	829	36	137	193	35	100
C-51	795	1349	1114	411	917	112	245	240	36	158
C-52	238	214	212	28	173	0	3	35	48	22
M-1	45	29	7	3	21	0	0	13	2	4
C-22	206	343	736	40	331	12	18	67	30	32
C-43	128	536	566	16	312	0	14	20	0	8
C-44	238	359	484	51	283	0	12	60	1	18
C-32	91	19	78	17	51	0	0	8	0	2
C-39	343	488	281	137	312	31	187	46	36	75
C-40	26	55	20	23	31	0	26	31	2	15
PLGA	192	82	55	3	83	0	0	0	8	2
P-6 + PLGA	1382	1538	2581	178	1420	106	387	371	38	226
P-7 + PLGA	152	324	174	12	166	0	2	0	2	1
C-8 + PLGA	1367	2547	1490	286	1423	2182	2193	716	211	1325
C-24 + PLGA	1017	1380	1362	52	953	0	31	65	0	24
C-21 + PLGA	4000	1204	1870	325	1850	2959	2024	886	191	1515
C-42 + PLGA	1515	1417	2190	372	1374	425	1081	295	69	468
C-41 + PLGA	710	1940	1910	496	1264	535	1987	534	119	794
C-45 + PLGA	1380	2292	1920	634	1557	2408	4000	1693	642	2186
C-46 + PLGA	2201	2352	1432	472	1614	502	1309	257	100	542
C-47 + PLGA	3579	4000	1137	161	2219	46	271	30	0	87
C-50 + PLGA	2969	1209	1465	402	1511	1548	2818	1242	327	1484
C-51 + PLGA	2018	4000	1000	463	1870	1837	3241	1154	536	1692
C-52 + PLGA	1172	1726	1551	117	1142	12	34	34	0	20
M-1 + PLGA	215	159	23	3	100	0	1	0	0	0
C-22 + PLGA	4000	2975	1085	136	2049	325	1186	226	42	445
C-43 + PLGA	2210	2594	1354	194	1588	358	1293	402	49	526
C-44 + PLGA	1452	4000	2006	276	1934	986	4000	1768	192	1736
C-32 + PLGA	2211	4000	2759	133	2276	204	1142	771	12	532
C-39 + PLGA	1800	4000	2275	274	2087	2167	4000	2613	736	2379
C-40 + PLGA	1438	498	1813	160	977	2758	4000	1556	370	2171
SAC	1618	1271	1053	123	1016	285	110	57	0	113

*Example 37 Immunostimulatory Activity of CICs*

**[0501]** This example shows immunomodulation assays for additional linear CICs (some containing both phosphorothioate (PS) and phosphodiester (PO) linkages) and branched CICs (Tables 11 and 12). Comparison of C-94, a branched CIC, with C-21, a linear CIC containing the same nucleic acid moieties, showed that the branched CIC induced 4-fold more IFN- $\alpha$  than the linear CIC. The amounts of IFN- $\gamma$  and IFN- $\alpha$  induced were significantly increased for each CIC by formulation with cPLGA. The phosphodiester versions of C-94 and C-93 were active only when formulated. C-87 showed remarkable induction of IFN- $\alpha$ .

TABLE 11

stim	IFN- $\gamma$ (pg/ml)					IFN- $\alpha$ (pg/ml)				
	28042	28043	28044	28045	mean	28042	28043	28044	28045	mean
cells alone	11	4	0	13	7	8	2	3	64	19
P-6	324	1036	529	653	636	9	34	22	108	43
P-7	34	19	48	35	34	0	0	4	54	15
C-8	623	753	646	604	656	78	25	52	256	103
C-53	39	27	38	26	32	0	0	0	5	1
C-49	367	433	767	353	480	30	8	100	88	57
C-84	29	23	69	232	88	0	0	5	222	57
C-85	17	13	315	134	120	0	0	28	24	13
C-94	443	198	1417	888	736	302	252	664	1855	768
C-93	8	1	41	17	17	7	3	81	61	38
C-21	572	460	4000	1644	1669	146	94	191	349	195
C-9	691	268	590	1306	714	39	0	11	64	29
PLGA	9	5	59	72	36	7	0	98	112	54
P-6 + PLGA	601	358	1474	1941	1093	115	116	515	1298	511
P-7 + PLGA	13	13	46	65	34	5	0	0	43	12
C-8 + PLGA	284	551	1781	3113	1432	595	396	1013	2259	1066
C-53 + PLGA	21	12	217	210	115	19	0	0	42	15
C-49 + PLGA	1471	1219	4000	2061	2188	904	460	4000	1040	1601
C-84 + PLGA	235	232	291	956	428	1777	914	4000	3641	2583
C-85 + PLGA	313	294	554	1167	582	2116	921	4000	2413	2362
C-94 + PLGA	2412	755	4000	3379	2637	1883	1640	4000	4000	2881
C-93 + PLGA	880	316	869	1251	829	778	471	2045	988	1071
C-21 + PLGA	4000	690	4000	2533	2806	712	577	2572	1571	1358
C-9 + PLGA	1451	763	4000	1804	2005	389	199	397	477	366

TABLE 12

stim	IFN-g (pg/ml)					IFN-a (pg/ml)				
	28218	28219	28220	28221	mean	28218	28219	28220	28221	mean
cells alone	5	5	5	5	5	32	32	32	32	32
P-6	13	7	25	141	47	32	32	32	32	32
P-7	5	5	5	5	5	32	32	32	32	32
C-87	83	24	38	977	281	3075	32	4269	265	1910
C-94	15	39	44	269	92	32	167	633	412	311
SAC	2552	621	1383	647	1301	483	105	32	452	268

*Example 38 Position of Sequence Motif in CIC*

[0502] This example describes immunomodulation assays for a number of CICs (some of which were assayed in different donors in previous examples) and illustrates the effect of nucleic acid sequence position in a CIC.

[0503] The CICs tested included CICs containing two different CG-containing nucleic acid sequences in nucleic acid moieties (TCGTCGA and ACGTTCG) along with one nucleic acid moiety not containing a CG sequence (AGATGAT). Of the CG-containing nucleic acid sequences, CIC's containing a TCGTCGA sequence have greater activity than CIC's containing only ACGTTCG. Of these two, CICs with TCGTCGA were more active. The general structure of the CICs used in this example, N<sub>1</sub>-S<sub>1</sub>-N<sub>2</sub>-S<sub>2</sub>-N<sub>3</sub>, can be used to describe the placement of the motifs within the CIC. Placing the most active motif, TCGTCGA, in the N<sub>1</sub> position led to the most active CICs (C-8, C-56). Placement in the N<sub>2</sub> position also conferred activity. For instance, C-57 with the TCGTCGA in the N<sub>2</sub> position was somewhat more active than C-58, with the TCGTCGA in the N<sub>3</sub> position. A CIC with a ACGTTCG sequence in the N<sub>1</sub> position, while being less active than a similar CIC with a TCGTCGA sequence, was more active than a CIC with the sequence AGATGAT, in the N<sub>1</sub> position (compare C-57 and C-58 to C-59 and C-60). In this experiment, C-61, which contained nucleic acid moieties that comprise CG motifs, but not TCG motifs, induced IFN- $\gamma$  when formulated with cPLGA. See Table 13.



TABLE 13

stim	IFN- $\gamma$ (pg/ml)					IFN- $\alpha$ (pg/ml)				
	28156	28157	28158	28159	mean	28156	28157	28158	28159	mean
cells alone	125	3	4	5	34	3	3	1	8	4
P-6	1132	872	207	231	611	52	484	7	32	144
P-8	255	20	31	31	84	16	9	24	8	14
C-8	1612	742	340	197	723	102	755	61	160	270
C-9	1162	729	192	329	603	26	142	78	20	67
C-23	733	576	202	295	452	26	235	59	169	122
C-54	297	378	88	218	245	8	96	8	13	31
C-55	511	566	55	186	329	9	5	63	3	20
C-56	1223	543	203	563	633	98	415	57	131	175
C-57	419	323	67	262	268	5	52	61	42	40
C-58	404	288	59	84	209	13	30	29	23	24
C-60	304	209	26	38	144	5	22	3	1	8
C-61	92	179	35	63	92	3	0	47	0	13
PLGA	43	63	5	11	30	85	246	0	3	83
P-6 + PLGA	1070	2643	251	496	1115	582	2948	418	359	1077
P-8 + PLGA	95	115	26	34	67	43	8	2	23	19
C-8 + PLGA	1083	1862	269	1129	1086	4000	4877	857	1573	2827
C-9 + PLGA	814	1412	307	992	881	1398	1778	418	483	1019
C-23 + PLGA	825	865	182	1423	824	1020	1621	240	597	869
C-54 + PLGA	838	1150	157	1751	974	752	1265	147	278	611
C-55 + PLGA	1048	960	247	2356	1153	505	801	78	211	399
C-56 + PLGA	792	604	321	4000	1429	4000	4000	852	2433	2821
C-57 + PLGA	1027	814	101	3056	1250	555	1476	10	252	573
C-58 + PLGA	804	1065	135	1021	756	179	932	3	139	313
C-60 + PLGA	650	858	56	1014	645	71	118	32	50	68
C-61 + PLGA	1265	1508	238	864	969	4	80	1	63	37
SAC	780	1184	83	659	677	208	55	6	34	76

[0504] This experiment also compared immunomodulatory activity of two types of branched CICs: C-94 has HEG moieties between the branching glycerol component and the nucleic acid moieties, while C-28 has the nucleic acid moieties attached directly to the glycerol spacer. See Table 14. Interestingly, while the induction of IFN- $\gamma$  was similar for both branched CICs, the induction of IFN- $\alpha$  was dramatically higher for the CIC containing the HEG spacers. A branched CIC, containing three P-6 sequences attached via their 5-ends to a maleimido-activated triethylamine spacer (C-99), induced IFN- $\gamma$  only when formulated with cPLGA and did not induce IFN- $\alpha$ . In general, the greatest IFN- $\alpha$  production was produced using CICs with nucleic acid moieties attached via a branched structure and having multiple

unattached or “free” 5'-ends of nucleic acid moieties, and including spacers that provide conformational flexibility and distance between the nucleic acid moieties.

TABLE 14

stim	IFN- $\gamma$ (pg/ml)					IFN- $\alpha$ (pg/ml)				
	110	112	119	120	mean	110	112	119	120	mean
cells alone	44	24	20	28	29	20	200	2	2	56
P-6	1508	344	144	104	525	50	172	234	72	132
P-7	124	24	16	40	51	2	32	474	2	128
C-8	1152	540	136	48	469	196	30	264	42	133
C-59	256	52	28	40	94	2	6	2	2	3
C-63	1536	376	80	60	513	294	38	464	228	256
C-50	1096	264	52	48	365	716	84	838	636	569
C-51	1528	240	52	40	465	1408	72	2200	622	1076
C-45	880	192	52	36	290	446	130	1074	428	520
C-41	512	100	32	32	169	58	2	182	6	62
C-42	1508	204	56	56	456	250	26	156	36	117
C-46	1224	400	68	36	432	58	2	208	48	79
C-52	472	48	40	28	147	2	2	292	2	75
C-39	604	116	108	32	215	674	26	444	250	349
C-40	180	12	4	20	54	6	198	152	2	90
C-94	5168	284	104	120	1419	1608	144	2610	878	1310
C-28	5564	52	44	60	1430	38	4	56	26	31
C-99	276	12	16	40	86	22	4	86	2	29
PLGA	32	8	72	120	58	10	2	60	92	41
P-6 + PLGA	1640	968	960	2300	1467	948	260	1298	1470	994
P-7 + PLGA	72	16	32	316	109	14	14	22	2	13
C-8 + PLGA	1948	1220	1188	2384	1685	6674	1138	2130	2650	3148
C-59 + PLGA	680	824	620	1828	988	234	2	76	278	148
C-63 + PLGA	1208	1580	2340	2092	1805	4148	738	2796	2298	2495
C-50 + PLGA	812	3684	1432	992	1730	3768	1414	4161	3402	3186
C-51 + PLGA	1240	11216	2896	924	4069	5244	1260	5104	6148	4439
C-45 + PLGA	2736	3024	3056	2472	2822	5532	1544	5474	4206	4189
C-41 + PLGA	3168	1808	16000	3656	6158	3542	746	2074	2094	2114
C-42 + PLGA	1612	2032	10212	1908	3941	3462	1030	2118	2054	2166
C-46 + PLGA	3048	2012	3720	3608	3097	2372	638	2372	2682	2016
C-52 + PLGA	1032	1236	2344	1724	1584	64	20	252	206	136
C-39 + PLGA	2024	1332	8228	1244	3207	3764	846	3078	2658	2587
C-40 + PLGA	1360	1244	5364	1864	2458	2362	684	3794	2616	2364
C-94 + PLGA	2668	3188	8840	3396	4523	5658	1838	8000	6346	5461
C-28 + PLGA	2104	2568	3572	1320	2391	302	2	284	198	197
C-99 + PLGA	768	672	5316	472	1807	114	80	344	260	200

### Example 39 Activity of Branched CICs

[0505] This example demonstrates that branched CICs with multiple free 5'- ends and conformational flexibility provided by HEG spacers induced more IFN- $\alpha$  relative to linear CICs with HEG spacers (compare C-94 with C-21 and C-96 with C-23) or branched CICs without additional (HEG) spacers (compare C-94 with C-28 and C-96

with C-27). Adding another HEG spacer and a 4-base nucleic acid moiety to C-96 caused a reduction of IFN- $\alpha$  induction (compare C-96 with C-97). See Table 15.

[0506] Immunostimulatory activity of two CICs containing trimeric 5'-TCG-3' motifs was tested (C-91 and C-68). While neither CIC was active on its own, C-91 had significant activity when formulated on cPLGA.

[0507] A hydrophilic polyamide-containing STARBURST dendrimer® with multiple P-6 sequences conjugated to it (C-102), had significantly more IFN- $\alpha$  activity than the P-6 sequence alone, when compared with an equal amount of P-6 (on a P-6 strand per strand basis). This result confirms, using a different composition and synthetic protocol from that demonstrated above, the utility of a multimeric delivery of 5'-CG-3'-containing nucleic acid moieties on a flexible, hydrophilic core for significantly increased induction of IFN- $\alpha$ .

Table 15

stim	IFN-g (pg/ml)						IFN-a (pg/ml)					
	28185	28186	28187	28188	mean	x 4	28185	28186	28187	28188	mean	x 2
cells alone	5	1	13	1	5	20	36	4	32	4	19	38
P-6	205	17	148	8	94	378	120	41	4	4	42	84
P-7	0	4	19	2	6	25	4	25	5	31	16	32
C-8	154	25	123	9	78	311	196	31	202	4	108	217
C-94	181	61	384	17	161	644	1895	239	136	13	571	1142
C-28	162	24	75	5	66	266	14	4	0	4	6	11
C-21	244	37	125	7	103	413	443	64	1	4	128	256
C-23	42	14	29	3	22	88	83	27	59	55	56	112
C-27	49	3	21	3	19	75	4	4	39	4	13	25
C-96	163	22	195	12	98	392	2550	446	118	40	788	1577
C-97	259	16	125	5	101	405	307	71	4	2	96	192
C-9	189	24	95	11	80	319	25	16	4	146	48	95
C-86	1	4	30	5	10	40	7	4	4	31	11	22
C-91	3	4	6	7	5	20	9	46	37	4	24	48
C-68	0	4	2	1	2	7	4	13	25	4	11	23
C-102	158	43	101	6	77	307	1880	187	109	4	545	1090
PLGA	10	4	13	4	8	30	4	4	0	4	3	6
P-6 + PLGA	315	64	128	39	137	546	710	116	78	4	227	454
P-7 + PLGA	7	3	15	2	7	27	4	4	4	9	5	10
C-8 + PLGA	319	127	242	24	178	712	1599	646	601	35	720	1441
C-94 + PLGA	391	118	280	34	206	823	6761	19553	3949	207	7618	15235
C-28 + PLGA	395	65	175	13	162	649	84	145	15	13	64	128
C-21 +	333	49	177	20	145	579	3581	3169	1340	64	2038	4077

PLGA												
C-23 + PLGA	199	67	102	15	96	382	599	250	110	21	245	490
C-27 + PLGA	292	170	95	14	142	570	54	58	4	39	39	78
C-96 + PLGA	400	186	244	41	218	872	27504	5572	2464	173	8928	17857
C-97 + PLGA	356	177	124	39	174	696	2264	668	285	48	816	1632
C-9 + PLGA	384	82	93	19	145	579	479	451	193	35	290	579
C-86 + PLGA	11	3	84	4	25	101	33	4	4	4	11	22
C-91 + PLGA	161	101	114	1	94	377	880	494	316	4	423	847
C-68 + PLGA	31	8	24	4	17	67	14	51	4	4	18	37
C-102 + PLGA	774	132	380	7	323	1293	2094	397	221	26	684	1369
SAC	195	22	274	15	127	506	73	4	151	102	82	165

#### Example 40

[0508] This experiment examined the activity of a series of CICs containing a hexameric nucleic acid motif, 5'-TCGTCG-3', and multiple spacers attached to the 3'-end of the nucleic acid moiety (C-13, C-14, C-15 and C-16). See Table 16. None of the CICs was active when used alone, however all had significant activity when formulated on cPLGA.

Table 16

stim	IFN- $\gamma$ (pg/ml)					IFN- $\alpha$ (pg/ml)				
	28057	28058	28059	28060	mean	28057	28058	28059	28060	mean
cells alone	1	2	1	39	11	0	0	0	0	0
P-6	83	103	1230	85	375	621	396	145	123	321
P-7	1	2	3	4	2	0	0	0	0	0
C-13	2	3	12	5	6	31	0	249	0	70
C-14	3	1	1	3	2	0	0	0	0	0
C-15	5	3	0	1	2	0	0	0	0	0
C-16	1	2	0	6	2	0	0	0	0	0
PLGA	40	32	49	254	94	35	222	41	0	74
P-6 + PLGA	2000	2000	2000	452	1613	2000	2000	1747	403	1537
P-7 + PLGA	40	271	16	40	92	0	527	161	108	199
C-13 + PLGA	2000	262	221	168	663	5865	7994	5912	1437	5302
C-14 + PLGA	2000	359	732	2000	1273	3937	6871	6371	2953	5033
C-15 + PLGA	2000	585	2000	258	1211	2991	6282	4138	1731	3786
C-16 + PLGA	172	207	277	71	182	1842	2529	2333	1362	2017
SAC	673	2000	2000	2000	1668	920	2000	387	146	863

*Example 41: Effects of CICs in B-Cell Proliferation Assay*

[0509] Human PBMCs were isolated from heparanized blood from two normal subjects. Some PBMCs were held in reserve while the remainder was incubated with CD19+ MACS beads (Miltenyi Biotec). These cells were then passed through a magnet, separating the CD19+ B cells through positive selection. This population was >98% CD19+ as determined by FACS analysis. B cells were then cultured at  $1 \times 10^5/200 \mu\text{l/well}$  in 96-well round-bottomed plates. In some cases, PBMCs were also cultured, but at  $2 \times 10^5/200 \mu\text{l/well}$ . Cells were stimulated in triplicate with  $2 \mu\text{g/ml}$  polynucleotide or CIC. The culture period was 48 hours at  $37^\circ\text{C}$ . At the end of the culture period, the plates were pulsed with  $^3\text{H}$ -thymidine,  $1 \mu\text{Ci/well}$ , and incubated for an additional 8 hours. Then the plates were harvested using standard liquid scintillation techniques and data was collected in counts per minutes (cpm).

[0510] Experiment A: The results of Experiment A (Table 17) demonstrate that polynucleotides (P-6) and CICs (C-8, C-9, C-21, C-28) containing 5'-C,G-3' motifs cause B cells to proliferate. Control compounds, P-7 and M-1, and a heptameric polynucleotide, P-1, generated little to no B cell proliferation. The branched CIC, C-28, and the CIC containing the propyl spacers, C-9, induced more B cell proliferation than CICs containing hexaethylene glycol spacers, C-8 and C-21. The proliferation of PBMCs mirrored that of B cells.

Table 17

cell type	stim	Donor 146				Donor 147				mean
		cpm1	cpm2	cpm3	mean	cpm1	cpm2	cpm3	mean	of both
B cells	cells alone	538	481	795	605	482	360	296	379	492
B cells	P-6	29280	33430	30056	30922	35729	18032	21166	24976	27949
B cells	P-7	4858	5810	7079	5916	4364	4066	2774	3735	4825
B cells	P-1	761	608	721	697	569	460	687	572	634
B cells	C-8	23815	30066	22969	25617	20914	22370	23659	22314	23966
B cells	C-9	35365	42705	45231	41100	55543	49035	44985	49854	45477
B cells	C-21	28467	16074	19258	21266	17604	18851	19887	18781	20024
B cells	M-1	1514	2815	1173	1834	1679	1667	1436	1594	1714
B cells	C-28	50999	54630	46418	50682	65593	51040	50357	55663	53173
PBMCs	cells alone	2744	2303	2284	2444	1301	2402	2143	1949	2196
PBMCs	P-6	22067	23740	28099	24635	26436	23830	17531	22599	23617
PBMCs	P-7	7620	8362	9686	8556	9783	9841	10476	10033	9295
PBMCs	P-1	9724	3041	2425	5063	1706	1960	324	1330	3197
PBMCs	C-8	47202	40790	44811	44268	38845	39733	27981	35520	39894
PBMCs	C-9	55348	24857	39953	40053	88106	65413	90665	81395	60724
PBMCs	C-21	30338	22685	22383	25135	28819	530	37088	22146	23641
PBMCs	M-1	8753	5203	4496	6151	1034	3298	1674	2002	4076
PBMCs	C-28	94977	121595	84977	100516	103916	91439	100905	98753	99635

[0511] Experiment B : Experiment B (Table 18) evaluated the effects of the spacer composition, as well as the CIC structure (linear vs. branched), on B cell proliferation. Linear CICs containing propyl, butyl, abasic, and hydroxymethylethyl spacers tended to induce more B cell proliferation than the corresponding CICs containing either hexaethylene glycol or triethylene glycol spacers (compare C-10, C-11, C-17, C-18, C-20, C-25). The dodecyl spacer rendered the CIC inactive (C-19). Notably, the B cell proliferation data does not necessarily mirror the cytokine data shown above, with particular differences see between B cell proliferation and IFN- $\alpha$  induction.

TABLE 18  
PROLIFERATION ASSAY

sample	cell	stim	121				194				mean of both
			cpm1	cpm2	cpm3	mean	cpm1	cpm2	cpm3	mean	
1	B cells	cells alone	451	757	297	502	203	228	151	194	348
2	B cells	P-6	19996	15031	19804	18277	13678	12732	9003	11804	15041
3	B cells	P-7	1623	1821	2901	2115	1992	1593	1686	1757	1936
4	B cells	C-8	2604	12078	17696	10793	9333	9391	7602	8775	9784
5	B cells	C-9	21938	35400	23877	27072	13660	16717	17866	16081	21576
6	B cells	C-10	15142	14136	16158	15145	7480	5458	5943	6294	10720
7	B cells	C-11	30367	30412	18528	26436	16967	20898	11253	16373	21404
8	B cells	C-22	17147	14014	6844	12668	6472	5540	3894	5302	8985
9	B cells	C-94	11418	14406	11110	12311	7361	8505	5349	7072	9692
10	B cells	C-28	35393	26954	26780	29709	21588	13691	15691	16990	23350
11	B cells	C-17	27975	30426	9895	22765	17467	14890	10518	14292	18529
12	B cells	C-18		17085	14653	15869	10028	12217	10538	10928	13398
13	B cells	C-19	858	1099	926	961	371	403	312	362	662
14	B cells	C-20	31276	30851	28532	30220	18082	18705	17481	18089	24155
15	B cells	C-23	10628	16221	20087	15645	8730	6532	9596	8286	11966
16	B cells	C-24	8206	6789	2799	5931	3979	3407	3468	3618	4775
17	B cells	C-25	34360	35016	26480	31952	16060	19509	17384	17651	24802

*Example 42: Immunomodulation of Mouse Cells by CIC*

[0512] Polynucleotides and chimeric compounds were tested for immunostimulatory activity on mouse splenocytes. Immunostimulation was assessed by measurement of cytokine secretion into the culture media. Cytokine levels in the culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) tests.

[0513] Cells were isolated and prepared using standard techniques. Spleens of 8 to 20 week-old BALB/c mice were harvested and the splenocytes isolated using standard teasing and treatment with ACK lysing buffer from BioWhittaker, Inc. Four spleens were pooled in this experiment. Isolated cells were washed in RPMI 1640 media supplemented with 2% heat-inactivated fetal calf serum (FCS), 50  $\mu$ M 2-mercaptoethanol, 1% penicillin-streptomycin, and 2 mM L-glutamine and resuspended at approximately  $7 \times 10^5$  cells/ml in 10% FCS/RPMI (RPMI 1640 media with 10% heat-inactivated FCS, 50  $\mu$ M 2-mercaptoethanol, 1% penicillin-streptomycin, and 2 mM L-glutamine).

[0514] Cell cultures were set up in triplicate with approximately  $7 \times 10^5$  cells/well in a 96-well flat microtiter plate in 100  $\mu$ l 10%FCS/RPMI with the cells allowed to rest for at least 1 hour after plating. The indicated test compounds were incubated (at the indicated concentrations) for 24 hours at 37°C. Cell supernatants were harvested and frozen at -80°C. Cytokine production by the cells was determined by ELISAs, as shown in 19.

TABLE 19

Test Compound	Dose	IL-6	IL-12	IFN $\gamma$
P-6	5.0 $\mu$ g/ml	9311	5374	2505
	1.0 $\mu$ g/ml	5760	4565	2175
	0.1 $\mu$ g/ml	121	1665	187
C-10	5.0 $\mu$ g/ml	3342	2329	199
	1.0 $\mu$ g/ml	1761	1738	104
	0.1 $\mu$ g/ml	9	122	9
C-11	5.0 $\mu$ g/ml	10098	4279	3342
	1.0 $\mu$ g/ml	11814	4914	3220
	0.1 $\mu$ g/ml	458	3359	960
P-7	5.0 $\mu$ g/ml	9	177	23
	1.0 $\mu$ g/ml	7	143	30
SAC		734	1343	18843
media alone		9	124	9

*Example 43: Induction of Immune-Associated Genes in the Mouse Lung After Intranasal Treatment with CICs.*

[0515] The ability of C-9, C-23, and P-6 (positive control) to induce mRNA expression of 75 different genes in the mouse lung was investigated. The genes evaluated included genes encoding cytokines, chemokines, cell surface molecules, transcription factors, metalloproteases, and other molecules. The study was performed at Northview Pacific Laboratories (Hercules, CA) with 6-8 week old female BALB/c mice from Jackson Labs (Bar Harbor, ME). Five mice per group were intranasally treated under light isoflurine anesthesia with 20  $\mu$ g of C-9, C-23, P-6 (positive control) or P-7 (negative control) in 50  $\mu$ L of saline. Previous experiments demonstrated that optimal induction of most genes was at 6 hrs after



treatment. Therefore, at 6 hrs the lungs were harvested and snap-frozen in liquid nitrogen and stored at -80°C for later use. Total RNA was isolated using RNeasy mini kits (Qiagen Inc., Valencia, CA). The RNA samples were DNase-treated (Roche Diagnostics, Mannheim, Germany) and converted into cDNA using Superscript II Rnase H-Reverse Transcriptase (Invitrogen, Rockville MD) as described in Scheerens et al., 2001, *Eur. J. of Immunology* 31:1465-74. The cDNA samples were pooled per group and in each pooled sample the expression of mRNA of 75 genes was measured using real-time quantitative PCR (ABI Prism 5700, Perkin Elmer Applied Biosystems) and syber green (Qiagen Inc.). In addition to the genes of interest, in each sample the mRNA expression of a housekeeping gene was measured (HPRT or ubiquitin). In order to correct for the amount of RNA in each sample, all data were calculated relative to the expression of the housekeeping gene. A selection of the most upregulated genes is shown in Figure 5, with data expressed as fold-induction over the response in control-treated (P-7) mice. The data demonstrate that C-9, C-23, and P-6 potently induce the expression of a variety of genes including IL-6, IL-12p40, IFN-alpha, IP-10, and IL-10. Treatment of mice with C-9, however, induced considerably higher mRNA expression of IFN-alpha when compared to the C-23 or P-6 treated group.

*Example 44: In Vivo Activity of CICs*

[0516] An *in vivo* study was performed by injecting mice (10 mice/group) subcutaneously in the scruff of the neck with 20 ug (200 ul volume) of P-6 (positive control), P-7 (negative control), C-9, C-23, P-1 or P-11. Blood was collected 2 hours later. For the LPS positive control group, mice were injected intraperitoneally with a 200 ul volume, and blood was collected 1.5 hours later (i.e., at the peak of LPS induced TNF- $\alpha$  activity). The blood was clotted and the serum was prepared and stored at -80°C until assayed. Serum cytokines were assayed using Biosource

cytoscreen kits for TNF- $\alpha$  and Pharmingen antibody pairs for mIL-6 and mIL-12.

All samples were assayed in duplicate.

[0517] P-6 and the two CICs, C-9 and C-23, each induced IL-12 p40, IL-6, and TNF- $\alpha$ , while the control oligonucleotide, P-7, was inactive (Figure 6A-C). CIC C-23 was more potent than C-9 and P-6 in this assay. As expected, the hexamer (P-11: 5'-AACGTT) and heptamer (P-1: 5'-TCGTCGA) were inactive.

*Example 45: Primate Immune Response to Antigen and CICs*

[0518] Immune responses to administration of hepatitis B surface antigen (HBsAg) in the presence of CICs were examined in baboons.

[0519] HBsAg was recombinant HBsAg produced in yeast. Groups of baboons (eight animals per group) included male and female baboons with weights ranging from 8-31 kg (group mean weights at 13-16 kg) at the start of the study.

[0520] The baboons were immunized two times, at a two-month interval (0 and 2 months), by intramuscular injection (IM) with 20  $\mu$ g HBsAg in a 1 ml volume. As outlined below, some of the groups also received CICs (C-8 or C-9) or a positive control (P-6) with the HBsAg.

[0521] Bleeds on all animals were collected prior to immunization and at 2 weeks post-immunization. Anti-HBsAg IgG titers were measured as follows. Baboon serum samples were analyzed by AUSAB EIA commercial kit (Abbott Labs Cat. # 9006-24 and 1459-05) using human plasma derived HBsAg coated beads. Samples were tested along with a panel of human plasma derived HBsAg positive and negative standards ranging from 0-150 mIU/ml. Biotin conjugated HBsAg and rabbit anti-biotin-HRP conjugated antibody was used as the secondary antibody complex used for detection. The assay was developed with ortho-phenylenediamine (OPD) and the absorbance values were determined at 492 nm with background subtraction at 600 nm (Quantum II spectrophotometer, Abbott Labs). Using the specimen absorbance value the corresponding concentration of anti-HBsAg is expressed in

milli-international units per ml (mIU/ml) as determined from the standard curve according to parameters established by the manufacturer. For diluted specimens, quantitation was based on the specimen absorbance that resulted in a value between 0-150 mIU/ml, multiplying by the dilution factor to arrive at the final concentration.

[0522] Statistical analysis was done with log-transformed data by analysis of variance (NCSS97 Statistical Software program, Kaysville, UT) using One-Way ANOVA Planned Comparison ( $\alpha = 0.05$ ).  $p \leq 0.05$  was considered significant.

The animal groups tested were immunized as follows:

Group 1 - 20  $\mu$ g HBsAg;

Group 2 - 20  $\mu$ g HBsAg + 1000  $\mu$ g P-6;

Group 3 - 20  $\mu$ g HBsAg + 1000  $\mu$ g C-8;

Group 4 - 20  $\mu$ g HBsAg + 1000  $\mu$ g C-9

[0523] Results from the study are shown in Table 20 below. Administration of CICs or the positive control P-6, in conjunction with HBsAg resulted in increased titers of anti-HBsAg antibodies as compared to administration of HBsAg alone. In a pairwise comparison, the immune response detected in Groups 2, 3, and 4 were significantly different from that detected in Group 1 ( $p < 0.05$  for Group 2 and  $p < 0.005$  for Groups 3 and 4, post-second immunization). There was no statistical difference found between groups 2, 3, and 4.

**TABLE 20**  
**Baboons Antibody Response (AUSAB EIA)**  
**HBsAg + CIC**

#	Group # Vaccine	Anti-HBsAg (mIU/ml) post-first	post-second
B339	1 HBV (20 ug)	0	7
B340		0	63
B341		0	15
B342		0	80
B343		0	55
B344		0	50
B345		0	28
B346		0	24
	Mean	0	40
	Stdev	0	26
B347	2 HBV (20 ug) P-6 (1000 ug)	0	329
B348		6	121
B349		0	108
B350		17	13,569
B351		0	315
B352		0	38
B353		15	1,446
B354		21	1,675
	Mean	7	2200*
	Stdev	9	4,637
B379	3 HBV (20 ug) C-8 (1000 ug)	2	184
B380		0	3,038
B381		0	41,706
B382		125	3,718
B383		0	250
B384		52	13,750
B385		0	11,626
B386		0	79
	Mean	22	9294**
	Stdev	45	14,121
B387	4 HBV (20 ug) C-9 (1000 ug)	0	5,605
B388		42	8,978
B389		0	312
B390		0	2,992
B391		405	12,663
B392		26	112
B393		75	2,364
B394		0	52
	Mean	68	4135**
	Stdev	139	4,633

\* p<0.05, \*\* p<0.005 compared to HBV alone (Group 1)

*Example 46: In Vivo Responses Generated by a CIC- Antigen Conjugate*

**[0524]** This example shows the induction of an antibody-mediated immune response in mice by administration of a CIC-antigen conjugate.

[0525] As described below, 10 mice/group were immunized twice intradermally (in the tail) at two week intervals with C-11/Amb a 1 conjugate synthesized as described below (1ug or 10 ug), P-6/Amb a 1 (1 ug) or Amb a 1 (1 ug). Anti-Amb a 1-specific IgG1 and IgG2a titers were determined from sera taken 2 weeks post each injection. *In vitro* re-stimulations were done on spleen cells at 6 weeks post 2<sup>nd</sup> immunization to determine Amb a 1-specific IFN $\gamma$  and IL-5 responses.

[0526] Mice immunized with the C-11-Amb a 1 conjugate showed the characteristic immune response pattern seen with the P-6-Amb a 1 reference material, specifically, a switch from a Th2, toward a Th1-type Amb a 1-specific immune response. Mice immunized with either the C-11 or P-6 conjugates developed strong IgG2a responses and reduced IgG1 responses. The conjugate treated groups also demonstrated a shut down of the IL-5 response and elevation of the IFN $\gamma$  response. Additionally, the immune responses to the C-11-Amb a 1 conjugate appear to increase in a dose dependant fashion, as demonstrated by comparing the 1 ug and 10 ug dose groups. The C-11-Amb a 1 conjugate elicits an immune response of similar quality to that seen with P-6-Amb a 1.

[0527] Results are shown in Tables 21-23.

#### General Procedure

[0528] The animal study was performed at Northview Pacific Laboratories (Hercules, CA) using 8-12 week old female BALB/c mice from Charles River Laboratories (Hollister, CA). 10 mice/group were injected twice intradermally in the tail (ID) at two-week intervals with one of the following materials: C-11/Amb a 1 conjugate (1 ug), C-11/Amb a 1 conjugate (10 ug), P-6/Amb a 1 conjugate (1 ug) or Amb a 1 antigen (1 ug). Bleeds were collected via the retro-orbital route two weeks after each of the injections and serum prepared for antibody determination. Six weeks after the 2<sup>nd</sup> injection spleens were harvested for *in vitro* re-stimulation assays to determine cytokine response of IFN $\gamma$  and IL-5. Spleens were assayed individually. Amb a 1 was used at 25 and 5 ug/ml for re-stimulation with  $5 \times 10^5$  cells/well and

supernatants harvested on Day 4 and stored at -80°C until assayed. Controls for the *in vitro* assay included SAC at 0.01% and PMA/IO at 10 ng/ml and 1 uM, respectively.

#### Mouse anti-Amb a 1 IgG1 and IgG2a Assays

**[0529]** Mouse serum samples were analyzed by ELISA in 96-well round-bottom plates that were coated with 50 µl/well Amb a 1 antigen at 1 µg/ml. Goat anti-mouse IgG1 (or IgG2a) biotin conjugated antibody was used as the secondary antibody. Streptavidin-horseradish peroxidase conjugate was used for detection. The assay was developed with TMB and the absorbance values were determined at 450 nm with background subtraction at 650 nm (Emax precision microplate reader, Molecular Devices, Sunnyvale, CA). The titer was defined as the reciprocal of the serum dilution that gave an ELISA absorbance of 0.5 OD using 4-parameter analysis (Softmax Pro97, Molecular Devices, Sunnyvale, CA). All samples were tested in duplicate wells on separate plates, and the titers were reported as the mean of the two values.

#### Mouse IL-5 and IFN-gamma Assays

**[0530]** Supernatants were tested for IL-5 and IFN $\gamma$  levels by capture ELISA on anti-cytokine monoclonal antibody coated plates. Biotinylated anti-cytokine MAbs were used as secondary antibodies. Streptavidin-horseradish peroxidase conjugate was used for detection and the assay was developed with TMB. Concentration was calculated from a standard curve assayed on each plate. The absorbance values were determined at 450nm with background subtraction at 650 nm (Emax precision microplate reader, Molecular Devices, Sunnyvale, CA). All samples were tested in duplicate wells on separate plates, and the concentrations were reported as the mean of the two values.

**[0531]** Statistics were done on log transformed data with the NCSS97 program (NCSS Statistical Software, Kaysville, Utah) using One-Way ANOVA with Planned

Comparisons,  $\alpha = 0.05$ . For the following study,  $p < 0.05$  is considered statistically significant.

#### Synthesis of the C-11/Amb a 1 Conjugate

##### Synthesis of activated C-11 (C-111):

**[0532]** The 5'-disulfide-C-11 (C-110) was dissolved in activation buffer (100 mM sodium phosphate/150 mM sodium chloride/pH 7.5) and activated by reduction with TCEP. The activated C-11 (C-111) was purified using a 5 ml Sephadex G25 column (Pharmacia) using the same activation buffer as mobile phase. Fractions were collected manually at 0.5-minute intervals starting at baseline rise. After purification, the concentration of the various fractions was determined using A260 and an extinction coefficient of 25.6 OD/mg.

##### Synthesis of activated Amb a 1:

**[0533]** Amb a 1 was activated by first blocking the its free-sulphydryls, and then adding a hetero-functional cross-linker. Excess reagents were removed by desalting using a HiTrap G-25 desalting column (Pharmacia Catalog #17-1408-01). The resulting activated Amb a 1 had an average of 9.3 sites per protein activated.

##### Synthesis of the C-11/Amb a 1 Conjugate:

**[0534]** The activated C-11 (C-111) and activated Amb a 1 were combined and the resulting C-11/Amb a 1 conjugate was fractionated using a Superdex 200 size exclusion chromatography column (Pharmacia Cat. # 17-1088-01; 1 cm x 30 cm). Formulation buffer (10 mM phosphate, 150 mM NaCl, pH 7.2) was used as mobile phase. Fractions were collected at 1-minute intervals, starting when the baseline began to rise.

**[0535]** The conjugate samples were analyzed by SDS-PAGE using a 4-12% NuPAGE gel (Invitrogen, Catalog #NP0322) using MOPS buffer (Invitrogen, Catalog #NP0001), and by Size Exclusion Chromatography (SEC-HPLC) using a BioSep SEC-S3000 column (Phenomenex, Catalog #00H-2146-E0). After SDS-PAGE the protein was visualized by using Coomassie blue stain (GelCode, Pierce

Catalog #24596). Presence of the CIC was confirmed by using DNA-Silver stain (Pharmacia, Catalog #17-6000-30). Both SDS-PAGE and SEC-HPLC were used to define pooling criteria, and for characterization of the obtained pool. Protein concentration was measured by the Bicinchoninic acid method (BCA, Sigma Catalog #BCA-1).



TABLE 21  
Activity of C-11/Amb a 1 Conjugate in Mice  
IgG1 and IgG2a anti-Amb a 1 titers

Group	Animal #	Immunization	2 weeks post 1st Imm		2 weeks post 2nd Imm	
			IgG1	IgG2a	IgG1	IgG2a
1	1	C-11 / Amb a 1 conjugate	30	148	7,900	19,886
	2		30	221	13,037	19,735
	3		30	943	946	23,918
	4		30	64	5,485	10,487
	5	(1 ug)	38	1,894	3,805	9,945
	6		30	943	600	5,249
	7	ID	30	570	10,337	20,156
	8		30	259	600	8,350
	9		56	30	2,575	5,747
	10		30	30	8,381	28,971
mean			33**	510	5,367**	15,244*
std dev			8	599	4,400	8,285
2	11	C-11 / Amb a 1 conjugate	51	345	8,982	27,877
	12		30	667	201,008	612,739
	13		77	445	6,739	86,672
	14		30	1,662	22,578	121,770
	15	(10 ug)	30	67	190,835	88,745
	16		30	450	5,971	17,600
	17	ID	55	1,137	29,646	105,398
	18		99	1,119	70,159	183,152
	19		99	8,227	80,052	250,206
	20		30	1,613	6,235	63,616
mean			53*	1,573	62,221	155,778*
std dev			29	2,399	75,298	174,925
3	21	P-6/Amb a 1 reference conjugate	30	37	3,437	65,306
	22		1,422	303	15,652	6,198
	23		485	265	84,927	177,281
	24		170	1,182	37,379	56,074
	25	(1 ug)	903	2,027	38,121	76,572
	26		88	2,298	32,499	240,098
	27	ID	33	321	3,011	24,404
	28		30	55	24,307	20,796
	29		113	89	43,060	19,586
	30		30	39	37,116	7,317
mean			330	662	31,951	69,363
std dev			475	862	23,568	78,697
4	31	Amb a 1	3,405	349	172,827	6,244
	32		7,331	30	164,673	1,003
	33		2,847	35	112,766	7,174
	34		4,021	30	100,281	1,399
	35	(1 ug)	8,333	212	156,037	4,969
	36		1,214	286	118,407	2,125
	37	ID	1,279	30	396,404	600
	38		4,332	80	187,335	4,599
	39		569	30	63,536	600
	40		2,696	30	161,039	902
mean			3,603**	111*	163,331**	2,962**
std dev			2,554	123	90,406	2,530

a value of 30 was used for samples <30 post 1st immunization

a value of 600 was used for samples <600 post 2nd immunization

\*p<0.05, \*\*p<0.005 compared to P-6/Amb a 1

TABLE 22  
Activity of C-11/ Amb a 1 Conjugate in Mice  
In vitro IFN $\gamma$  response (pg/ml)

Animal	Immunization	Amb a 1		PMA	Media Alone
		25 ug/ml	5 ug/ml		
1	C-11 / Amb a 1 conjugate (1 ug)  ID	22501	16320	12505	45
2		16291	10054	8433	45
3		8534	5084	5835	45
4		16925	8322	3796	45
5		23136	11298	9225	45
6		24900	25489	21525	185
7		4383	2716	8855	45
8		DEAD	DEAD	DEAD	DEAD
9		16088	6285	27722	45
10		25067	12431	28201	45
	Mean	17536	10889	14011	61
	StDev	7289	6839	9353	47
11	C-11 / Amb a 1 conjugate (10 ug)  ID	19994	13466	33702	45
12		50732	25103	36467	45
13		54752	28422	21770	123
14		96417	78017	24601	1305
15		83356	43505	20021	151
16		88018	51299	40604	829
17		87839	59079	31562	83
18		49763	28468	58062	211
19		102646	60332	32669	3366
20		61939	29505	53393	756
	Mean	65868**	39652	35576	394
	StDev	24860	20160	13331	455
21	P-6/Amb a 1 reference conjugate (1 ug)  ID	4275	3083	36550	45
22		4307	996	23742	45
23		40761	16956	15407	45
24		40764	23643	8961	118
25		35645	30164	15915	209
26		40895	31027	13355	308
27		25538	14349	15515	73
28		15884	12432	11623	45
29		4215	4608	71066	45
30		63276	46897	43680	734
	Mean	27556	18416	25581	167
	StDev	20099	14603	19548	218
31	Amb a 1 (1 ug)  ID	3016	2585	108000	452
32		1193	277	94345	104
33		5112	6239	97567	461
34		1301	251	89623	49
35		6879	2972	77808	56
36		1187	673	77299	89
37		4492	2840	89253	282
38		6170	3765	70169	187
39		2099	1152	108000	132
40		2209	3895	103131	309
	Mean	3366**	2465	91520	212
	StDev	2143	1915	13239	156

a value of 18 was used for values <18

values were not included in calculations since value for media alone was >3 stdev + average of all media alone values (ie. 2014 pg/ml)

\*\*p<0.005 compared P-6/Amb a 1 for 25 ug/ml restimulation

TABLE 23  
Activity of C-11/ Amb a 1 Conjugate in Mice  
In vitro IL-5 response (pg/ml)

Animal	Immunization	Amb a 1		PMA	Media Alone
		25 ug/ml	5 ug/ml		
1	C-11 / Amb a 1 conjugate (1 ug)	67	106	2202	41
2		49	53	2406	24
3		264	24	968	24
4		24	24	2979	24
5		68	45	2851	46
6	ID	104	121	2547	129
7	DEAD	24	24	3935	24
8		DEAD	DEAD	DEAD	DEAD
9		24	24	1383	24
10		24	203	1837	53
	Mean	68	63	2320	33
	StDev	86	63	948	12
11	C-11 / Amb a 1 conjugate (10 ug)	24	33	1655	24
12		41	73	2258	84
13		169	137	892	24
14		261	221	918	33
15		134	221	658	24
16	ID	253	187	778	29
17	DEAD	72	109	966	24
18		24	35	3656	24
19		334	231	1238	145
20		213	55	751	24
	Mean	153	130	1377	44
	StDev	111	80	940	40
21	P-6/Amb a 1 reference conjugate (1 ug)	24	24	1514	24
22		24	60	3725	24
23		157	277	1702	24
24		96	152	4473	24
25		88	36	1414	24
26	ID	39	372	1235	24
27	DEAD	218	176	1037	24
28		103	54	1382	24
29		24	459	2194	24
30		110	102	2990	24
	Mean	88	171	2167	24
	StDev	64	151	1174	0
31	Amb a 1 (1 ug)	724	331	1332	24
32		91	57	3186	24
33		930	1259	1574	56
34		375	674	2506	24
35		1093	763	2197	24
36	ID	1206	490	3715	27
37	DEAD	2808	2115	1727	67
38		1441	909	1655	58
39		1240	1228	1367	24
40		1373	481	1683	68
	Mean	1128**	831	2094	40
	StDev	734	588	808	20

a value of 24 was used for values <24

values were not included in calculations since value for media alone was >3 stdev + average of all media alone values (ie. 124 pg/ml)

\*p<0.05, \*\*p<0.005 compared to P-6/Amb a 1 for 25 ug/ml restimulation

#### Example 47: Effect of Spacer Moiety on CIC Activity

[0536] This example shows the effect of different spacer moieties on IFN- $\alpha$  induction. Comparison of C-90 (C3 CIC) and C-51 (HEG CIC) showed that C-51 induced 8-fold more IFN- $\alpha$  than C-90, although the amount of IFN- $\gamma$  induced by each CIC was similar. Similarly, comparison of branched CICs containing different linkers showed that for IFN- $\alpha$  induction, HEG (C-94) > TEG (C-103) > C3 (C-104) = no linker (C-28).

Table 24

stim	IFN- $\gamma$ (pg/ml) 28234	28235	28236	28237	mean	IFN- $\alpha$ (pg/ml) 28234	28235	28236	28237	mean
cells alone	4	4	4	4	4	16	16	16	16	16
P-6	15	52	51	1167	321	58	16	16	74	41
P-7	13	4	7	4	7	62	16	16	16	28
C-90	7	118	497	1586	552	16	46	117	345	131
C-51	17	123	193	1580	478	16	77	352	3798	1061
C-71	30	168	448	1663	577	17	30	538	1665	563
C-101	14	205	627	2612	865	16	249	1354	8566	2546
C-96	21	239	354	1396	503	16	120	608	993	434
C-97	10	119	269	980	345	16	16	140	16	47
C-100	27	183	490	1907	652	16	16	398	193	156
C-88	5	21	17	477	130	95	16	212	111	109
C-33	23	86	247	2076	608	16	16	16	91	35
C-21	4	107	308	1645	516	16	16	73	678	196
C-28	10	14	88	1229	335	16	16	16	16	16
C-94	7	161	239	1116	381	16	118	548	3631	1078
C-103	21	44	250	1854	542	16	21	126	213	94
C-104	14	4	87	125	58	16	29	16	16	19
PLGA	4	31	18	10	16	16	122	157	35	83
P-6 + PLGA	57	514	1052	3775	1350	16	694	1163	3444	1329
P-7 + PLGA	4	4	11	13	8	16	16	16	16	16
C-90 + PLGA	139	673	831	4618	1565	1175	696	4544	5103	2880
C-51 + PLGA	88	644	1064	3748	1386	3257	2168	8000	8000	5356
C-71 + PLGA	101	797	1254	3899	1513	3085	2244	8000	8000	5332
C-101 + PLGA	110	659	879	6944	2148	4679	4488	8000	8000	6292
C-96 + PLGA	143	1070	1167	5471	1963	4107	3237	6660	8000	5501
C-97 + PLGA	68	737	988	5327	1780	4742	4216	8000	8000	6240
C-100 + PLGA	176	1299	1742	7804	2755	1520	1092	4074	3777	2616
C-88 + PLGA	102	512	1148	5055	1704	803	613	2409	6412	2559
C-33 + PLGA	118	444	968	3947	1369	551	566	3514	6727	2840
C-21 + PLGA	159	411	1089	4056	1429	1369	1561	5366	8000	4074
C-28 + PLGA	28	131	1005	3868	1258	16	16	184	134	88
C-94 + PLGA	174	623	1352	4034	1546	4145	4653	7197	8000	5999
C-103 + PLGA	192	643	1388	5063	1822	895	1486	4456	5405	3061
C-104 + PLGA	40	73	641	4930	1421	16	16	128	92	63

*Example 48: Assessment of Isolated Immunomodulatory Activity of Polynucleotides Corresponding In Sequence to CIC Nucleic Acid Moieties*

[0537] This example further illustrates the immunostimulatory activity of CICs that contain nucleic acid moieties that do not have isolated immunomodulatory activity. The activity of polynucleotides corresponding in sequence to the CIC nucleic acid moieties were assayed alone or in combination with free spacers, and compared to the activity of a CIC containing the same amount of nucleic acid and spacer. For instance, 3 uM of CIC C-101 was compared with 9 uM P-14 or a mixture of 9 uM P-14 and 9 uM hexaethylene glycol and 3 uM glycerol (because C-101 contains three equivalents of P-14, three equivalents of hexaethylene glycol, and one equivalent of glycerol.) In all cases, the CICs were active while the short polynucleotides, both alone and mixed with spacers, were inactive. See Table 25. The activity of the spacers alone was tested at a concentration of 9 uM and all were completely inactive.

TABLE 25

stim	IFN-g (pg/ml)					IFN-a (pg/ml)				
	28250	28251	28252	28253	mean	28250	28251	28252	28253	mean
cells alone	18	5	9	1	8	16	16	30	19	20
P-6	121	18	34	37	52	16	16	16	16	16
P-7	104	1	39	1	36	16	16	16	16	16
Propyl spacer	13	1	1	1	4	16	16	16	16	16
Butyl spacer	8	5	1	1	4	16	16	16	16	16
Triethylene glycol	15	1	7	1	6	16	16	16	16	16
Hexaethylene glycol	12	1	1	15	7	16	16	16	35	21
Glycerol	16	12	2	1	8	16	16	16	16	16
C-51	135	45	164	167	128	181	246	95	1226	437
C-101	224	63	180	146	153	540	1472	509	2645	1291
P-14	10	34	11	10	16	16	16	16	16	16
P-14/HEG/glycerol	14	19	10	10	13	16	16	16	16	16
C-21	122	51	155	203	133	31	69	41	264	101
C-94	340	60	287	128	204	245	645	323	1198	603
P-1	54	21	56	1	33	16	16	16	16	16
P-1/HEG/glycerol	15	9	19	1	11	16	16	16	16	16
C-45	107	26	95	8	59	16	109	55	382	140
P-13	18	13	22	1	14	16	16	16	16	16
P-13/HEG	40	28	45	1	28	16	16	16	16	16
C-10	337	163	776	898	544	16	23	25	124	47
P-2	7	25	53	1	22	16	16	25	16	18
P-3	32	21	72	1	31	16	29	38	31	29
P-4	72	1	43	1	29	16	16	16	16	16
P-2/P-3/P-4/HEG	68	1	38	1	27	16	16	16	16	16

Example 49: Preparation of (5'-TCGACGT-3'-HEG)<sub>ave=185</sub>-Ficoll<sub>400</sub> (C-137)

#### A. Preparation of Maleimido-Ficoll<sub>400</sub>

[0538] Aminoethylcarboxymethyl (AECM)<sub>180</sub>-Ficoll<sub>400</sub> was prepared by the method of Inman (*J. Immunology*, 1975, 114: 704-709). On average there were 180 aminoethyl groups per mole of Ficoll (MW = 400,000 Da). 27.6 mg (62.6  $\mu$ mol) of sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate dissolved in 300  $\mu$ l of DMSO was added dropwise, with constant vortexing, to 23.2 mg (0.058  $\mu$ mol) of AECM<sub>180</sub>-Ficoll<sub>400</sub> dissolved in 1.0 ml of 0.1 M sodium phosphate buffer (pH 6.66). The reaction mixture was placed on a shaker for 2 h and then desalted on a Sephadex G-25 column to yield 20 mg of maleimido-Ficoll<sub>400</sub>. On average, there were approximately 165 maleimide groups per mole of Ficoll.

B. Preparation of 5'-TCGACGT-3'-HEG-(CH<sub>2</sub>)<sub>3</sub>-SH (C-136)

[0539] 5'-TCGACGT-3'-HEG-(CH<sub>2</sub>)<sub>3</sub>-SS-(CH<sub>2</sub>)<sub>3</sub>-OH (C-135) was synthesized analogously to C-116. To 10 mg (3.57  $\mu$ mol) of C-135 dissolved in 0.4 mL of 0.1 M sodium phosphate/150 mM sodium chloride/pH 7.5 buffer was added 5.7 mg (20  $\mu$ mol) of TCEP dissolved in 0.7 ml of the same buffer. The mixture was vortexed well and placed in a 40°C water bath for 2 h. The thiol (C-136) was purified by RP-HPLC (Polymer Labs PLRP-S column) using an increasing gradient of acetonitrile in triethylammonium acetate buffer (TEAA)/pH 7.0 and used immediately in the next reaction.

C. Preparation of (5'-TCGACGT-3'-HEG)<sub>x</sub>-Ficoll<sub>400</sub> (C-137)

[0540] To 5.5 mg (0.014  $\mu$ mol) of maleimido-Ficoll<sub>400</sub> dissolved in 0.7 ml of 0.1 M sodium phosphate/pH 6.66 was added 6.8 mg (2.5  $\mu$ mol) of C-136 dissolved in 3.45 mL of approximately 30% acetonitrile/TEAA/pH 7.0 buffer. The mixture was put on the shaker at RT overnight and the product was purified on a Superdex 200 column (Pharmacia). Calculations using the total weight of the isolated product and absorbance values at 260 nm showed the product contained, on average, approximately 185 oligonucleotides per mole of Ficoll. A second fraction containing a lower loading of oligonucleotides per mole of Ficoll was also obtained.

D. Activity of C-137

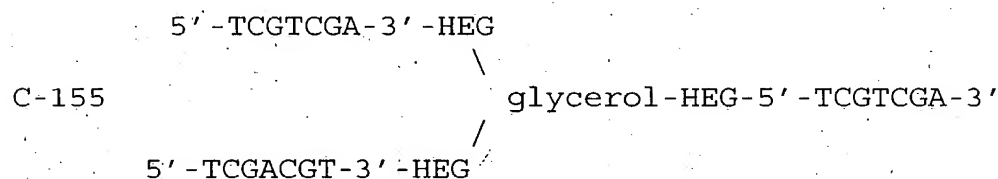
[0541] As shown in Table 26, the polysaccharide based CIC had striking activity in the cytokine response assays, in particular showing significant stimulation of IFN- $\alpha$ .

TABLE 26

Compound stim	IFN-g (pg/ml)						IFN-a (pg/ml)					
	28313	28314	28315	28316	mean	x 4	28313	28314	28315	28316	mean	
cells alone	1	9	11	11	8	32	31	122	100	98	88	
P6	1	38	47	309	99	395	31	130	122	134	104	
P7	1	11	12	20	11	43	31	176	107	121	109	
C-137	1	22	13	54	22	90	3612	5468	624	4000	3426	
SAC	87	77	56	4000	1055	4220	346	192	114	1172	456	

*Example 50: Synthesis of a CIC with a Branched Structure, Containing Two Different 5'-Nucleic Acid Moieties*

**[0542]** C-155, having the formula shown below, contains phosphorothioate linkages in the nucleic acid moieties, between the nucleic acid moieties and the HEG spacers, and between the HEG spacers and the glycerol branching spacer.



**[0543]** C-155 was synthesized as described in Example 17, with the following changes: The instrument was programmed to add the nucleic acid moieties and spacer moieties in the following order.

1. Use a 3'-support bound "A" solid support
2. Synthesis of 5'-TCGTCG-3'
3. Addition of HEG spacer phosphoramidite
4. Addition of asymmetrical branched phosphoramidite based on glycerol
5. Addition of HEG spacer phosphoramidite
6. Synthesis of 5'-TCGTCGA-3'
7. Detritylation and capping of the 5'-TCGTCGA-3' moiety
8. Removal of the levulinyl protecting group



9. Addition of HEG spacer phosphoramidite

10. Synthesis of 5'-TCGACGT-3'

[0544] The CIC was purified by RP-HPLC as described in Example 12 and characterized as described in Example 2.

*Example 51: Preparation of a Branched CIC with a Cage Structure Using Phosphoramidite Chemistry*

[0545] C-163, having the structure shown below and in Figure 9F, is synthesized as described in Example 20. All linkages are phosphorothioate.

C-163 (5'-CTGAACGTTTCAG-3'-HEG)<sub>3</sub>-trebler-HEG-5'-T-3'

(CTGAACGTTTCAG is SEQ ID NO:104). The three self-complimentary 12-mer nucleic acid moieties are hybridized to a second molecule of the CIC, as shown in Figure 9F, resulting in a cage structure. C-163, dissolved at a concentration of approximately 1.0 mg/ml in 50 mM sodium phosphate/150 mM sodium chloride/pH 7.2, is heated to 95°C for 3 min and then allowed to slowly cool in the heat block over a period of approximately 2 hours. The formation of the cage structure is confirmed by size exclusion chromatography.

*Example 52: Preparation of a Linear CIC with a Hairpin Structure*

[0546] C-159, having the structure shown below, is synthesized as described in Example 2 and purified by RP-HPLC, as described in Example 12. The linkages in the nucleic acid moieties and between the nucleic acid moieties and the HEG spacer are phosphorothioate.

C-159

5' -TGCGTGTAACGTTACACGCA-3' -HEG-5' -TGCGTGTAACGTTACACGCA-3'

(TGC GTGT AACGTTACACGCA is SEQ ID NO:114). In C-159, the first nucleic acid moiety is complementary to the second nucleic acid moiety and forms a hairpin structure when annealed in the presence of salt, as described in Example 51. C-160 is synthesized and annealed analogously.

*Example 53: Preparation of a Branched CIC with a Central Spacer Structure using a Conjugation Strategy*

[0547] C-140 was synthesized as shown in Figure 8G. To C-136 (7.3 mg, 2.7  $\mu$ mol), prepared as described in Example 49B, dissolved in 30% acetonitrile/0.1 M triethyl ammonium acetate/pH 7.0 (4.0 mL) was added 4-arm-bPEG-vinylsulfone (5.36 mg, 0.54  $\mu$ mol, MW = 10,000, Shearwater Polymers, Inc., Huntsville, AL) dissolved in 100 mM sodium phosphate/150 mM sodium chloride/pH 7.5 buffer (0.27 mL). The mixture was placed on a circular mixer overnight at room temperature and then purified on a Superdex 200 column (Amersham Pharmacia, Piscataway, NJ) using 10 mM sodium phosphate/150 mM sodium chloride/pH 7.2. C-139 was prepared analogously.

*Example 54: Preparation of Branched CICs with a Central Spacer Structure (C-168) and a Comb Structure (C-169) using Phosphoramidite Chemistry*

[0548] The structures of C-168 and C-169 are shown below and in Figures 8D and 8E. C-168 is synthesized as described in Example 17, with the following changes. C-168 contains phosphorothioate linkages in the nucleic acid moieties, between the nucleic acid moieties and the HEG spacers, and between the HEG spacers and the glycerol branching spacer.

C-168

5'-TCGTCGA-3'-HEG-[gly(HEG-3'-TGCAGCT-5')-HEG]<sub>3</sub>-5'-TCGAACG-3'

The instrument is programmed to add the nucleic acid moieties and spacer moieties in the following order.

1. Use a 3'-support bound "G" solid support
2. Synthesis of 5'-TCGAAC-3'
3. Addition of HEG spacer phosphoramidite
4. Addition of asymmetrical spacer phosphoramidite based on glycerol (gly)
5. Repeat steps 3 and 4 two more times
6. Addition of HEG spacer phosphoramidite
7. Synthesis of 5'-TCGTCGA-3'
8. Deprotect and cap the 5'-TCGTCGA-3'
9. Removal of the levulinyl protecting groups using a 90 min treatment with 0.5 M hydrazine hydrate in pyridine:acetic acid (1:1, v/v)
10. Addition of HEG spacer phosphoramidite
11. Synthesis of 5'-TCGACGT-3'

After removal of the 3 levulinyl protecting groups, as described in Step 9, the reagents are added in amounts 2-3 X the usual amounts because three nucleic acid moieties are being synthesized at one time. The CIC is purified by ion exchange chromatography using Source Q 30 (Amersham Pharmacia, Piscataway, NJ) as described in *Organic Process Research & Development* 2000, 4, 205-213.

[0549] C-169 is prepared analogously, except that Step 3' is inserted between Steps 3 and 4, where Step 3' is the synthesis of 5'-TTTTT-3' and Step 5 is the repetition of Steps 3' and 4 two more times.

C-169

5'-TCGTCGA-3'-HEG-(gly(HEG-3'-TGCAGCT-5')-5'-TTTTT-3')<sub>3</sub>-HEG-5'-TCGAACG-3'

*Example 55: Preparation of a Self-Assembling CIC Containing a Self-Complimentary Nucleic Acid Sequence That Can Form a Central Axis Structure*

**[0550]** The structure of C-167 is shown in Figure 9E and below. C-167 is synthesized as described in Example 19. C-167 contains phosphorothioate linkages in the nucleic acid moieties, between the nucleic acid moieties and the HEG spacers, and between the HEG spacers and the glycerol branching spacer.

C-167 (5'-TCGACGT-3'-HEG)<sub>2</sub>-glycerol-HEG-5'-TTGGCCAAGCTTGGCCAA-3'

The self-complimentary 18-mer nucleic acid moiety in the CIC is hybridized to a second molecule of the CIC, as shown in Figure 9E, by preparing a solution of C-167 at a concentration of approximately 1.0 mg/ml in 50 mM sodium phosphate/150 mM sodium chloride/pH 7.2, heating the solution to 95°C for 3 min, and then allowing the solution to slowly cool in the heat block over a period of approximately 2 hours. The formation of the double-stranded (central axis) CIC is confirmed by size exclusion chromatography.

*Example 56: Preparation of CICs with a Central Spacer Structure and an H-Structure (C-171) using Phosphoramidite Chemistry*

**[0551]** The structures of C-171 and C-170 are shown below and in Figures 8F and 8C. C-170 is synthesized as described in Example 17, with the following changes. C-170 contains phosphorothioate linkages in the nucleic acid moieties, between the nucleic acid moieties and the HEG spacers, and between the HEG spacers and the glycerol branching spacer.

C-170 (5'-TCGACGT-3'-HEG)<sub>2</sub>-glycerol-HEG-glycerol-(HEG-3'-TGCAGCT-5')<sub>2</sub>

The instrument is programmed to add the nucleic acid moieties and spacer moieties in the following order.

1. Use a 5'-support bound "T" solid support
2. Synthesis of 3'-TGCAGC-5' in the 5' to 3' direction (see Example 14)
3. Addition of HEG spacer phosphoramidite
4. Addition of asymmetrical branched phosphoramidite based on glycerol
5. Addition of HEG spacer phosphoramidite
6. Synthesis of 5'-TCGACGT-3' in the 3' to 5' direction
7. Detritylation and capping of the 5'-TCGACGT-3' moiety
8. Removal of the levulinyl protecting group with 0.5 M hydrazine hydrate in pyridine:acetic acid (3:2, v/v), 5 min
9. Addition of HEG spacer phosphoramidite
10. Addition of symmetrical branched phosphoramidite based on glycerol
11. Addition of HEG spacer phosphoramidite
12. Synthesis of 5'-TCGACGT-3' in the 3' to 5' direction

For Steps 11 and 12, 2X the usual amount of reagents are used because two chains are being synthesized simultaneously. This method results in a CIC with an central spacer structure. A second central spacer structure can be added to the first central spacer structure by addition of a second asymmetric branched phosphoramidite within one of the nucleic acid moieties. If an asymmetric branched spacer is used in Step 10, each nucleic acid moiety in the resulting CIC may contain a different sequence.

[0552] C-171 is synthesized analogously, except that Step 9 is the synthesis of 5'-TTTTT-3' instead of addition of the HEG spacer phosphoramidite. C-170 forms an H-structure.

C-171 (5'-TCGACGT-3'-HEG)<sub>2</sub>-glycerol-5'-TTTTT-3'-glycerol-(HEG-3'-TGCAGCT-5')<sub>2</sub>

*Example 57: Synthesis of Additional CICs*

**[0553]** Additional compounds described in Table 2, *supra* have been synthesized using the following methods:

Compound	Method of Synthesis
C-138	as described in Example 49
C-141	as described in Example 23
C-142, C-143, C-144, C-150, C-153, C-154, C-156, C-158	as described in Example 19
M-17-M-20	as described in Example 19, except that the appropriate spacers were inserted in place of the symmetrical spacer and/or the HEG spacer
C-151 and M-21	as described in Example 2, except that 6-amino-1-hexanol-CPG (AH-CPG; <i>Bioconjugate Chem.</i> 1992, <u>3</u> , 85-87; <i>Nucleic Acids Res.</i> 1993, <u>21</u> , 145-150) was used as the solid support in order to generate a 3'-aminohexyl linker on the CIC
C-152 and M-22	as described in Example 19, except that 6-amino-1-hexanol-CPG (AH-CPG; <i>Bioconjugate Chem.</i> 1992, <u>3</u> , 85-87; <i>Nucleic Acids Res.</i> 1993, <u>21</u> , 145-150) was used as the solid support in order to generate a 3'-aminohexyl linker on the CIC

**[0554]** Additional compounds described in Table 2, *supra*, are synthesized using the following methods:

C-166	as described in Example 19, except that the linkages are oxidized to phosphodiester linkages, as described in Example 25
C-159 and C-160	as described in Example 2
C-163	as described in Example 20
C-164	as described in Example 20, except that the linkages are oxidized to phosphodiester linkages, as described in Example 25
C-161, C-162, C-165	as described in Example 19

**[0555]** Compounds M-17 – M-22, among others described herein, do not include a CG motif and are used generally as controls in assays or experiments.

**[0556]** The remaining CICs and oligonucleotides (e.g., C-172, C-173-176, C-177, P-17, C-178, C-179-184, C-185-187, C-188-197, C-198-203, C-204-207, C-208-209, and M2-3) were synthesized analogously to those described herein.

[0557] CIC duplexes (e.g., C-202/C-203 duplex; C-208/C-209 duplex; C-202/C-209 duplex; C-203/C-208 duplex; and C-178 homoduplex) were prepared by annealing (1 mg/ml oligonucleotide heated 5 m at 95°C in PBS, allowed to cool slowly to room temperature, and stored at 4°C).

*Example 58: Induction of IFN- $\alpha$  Secretion by Multimeric CICs*

**[0558]** The ability of CICs and oligonucleotides to elicit IFN- $\alpha$  from human PBMCs was assayed as described in Example 28. The results shown below demonstrate that CICs that can self-hybridize (C-173, C-174, C-175) induce significantly more IFN- $\alpha$  from human PBMC than does the parent oligonucleotide (P-17) when used at low doses (e.g., 0.8 ug/ml). Each compound was assayed at three concentrations using PBMCs from four different individuals and the mean values determined.

Stim (amt, ug/ml)	IFN- $\alpha$ (pg/ml)				mean
	1	2	3	4	
medium	52	52	52	52	52
P-6 (20)	116	64	52	52	71
P-6 (4)	115	52	52	65	71
P-6 (0.8)	52	52	52	52	52
P-7 (20)	52	52	52	52	52
P-7 (4)	52	52	52	52	52
P-7 (0.8)	52	52	52	52	52
C-101 (20)	2330	109	215	157	703
C-101 (4)	52	52	59	204	92
C-101 (0.8)	52	52	52	52	52
M-3 (20)	52	52	52	52	52
M-3 (4)	52	52	52	52	52
M-3 (0.8)	52	52	52	52	52
C-173 (20)	14381	8060	66	299	5701
C-173 (4)	3828	2917	2197	3563	3126
C-173 (0.8)	272	1293	1138	2547	1312
C-174 (20)	1350	1176	61	837	856
C-174 (4)	5601	7845	1016	5895	5089
C-174 (0.8)	7907	11198	998	1752	5464
C-175 (20)	2250	732	52	52	771
C-175 (4)	7134	6779	906	1951	4193
C-175 (0.8)	21783	16605	851	2874	10528
P-17 (20)	1022	1792	52	52	729
P-17 (4)	9154	10388	531	837	5228
P-17 (0.8)	52	105	52	286	124



*Example 59: Induction of IFN- $\alpha$  Secretion by Multimeric CICs*

[0559] Assays were conducted as described for Example 58. The results shown demonstrate that CICs that hybridize to produce multimers with a total of four free 5'-ends with active TCG-containing heptamers (e.g., C-178 duplex, C-202/C-203 heteroduplex) induce significantly more IFN- $\alpha$  from human PBMC than CIC multimers containing only two free 5'-ends (C-101, C-202, C-203).

stim	IFN- $\alpha$ (pg/ml)				
	48	177	272	273	mean
medium	102	102	102	102	102
P-6 (20)	256	323	102	102	196
P-6 (4)	102	245	102	102	138
P-6 (0.8)	102	102	102	102	102
P-7 (20)	102	102	102	102	102
P-7 (4)	102	102	102	102	102
P-7 (0.8)	102	102	102	102	102
C-101 (20)	2212	4277	642	102	1808
C-101 (4)	818	3631	604	102	1289
C-101 (0.8)	102	102	185	102	123
M-3(20)	102	102	102	102	102
M-3 (4)	102	102	102	102	102
M-3 (0.8)	102	102	102	102	102
C-178 (20)	13946	19973	15050	3971	13235
C-178 (4)	16300	8304	1677	1732	7003
C-178 (0.8)	336	1150	149	102	434
C-202 (20)	1901	2742	612	339	1399
C-202 (4)	2250	2482	1222	215	1542
C-202 (0.8)	830	102	102	102	284
C-203 (20)	735	681	102	102	405
C-203 (4)	691	487	102	102	346
C-203 (0.8)	102	102	102	102	102
C-202/C-203 (20)	18550	17237	3474	2285	10386
C-202/C-203 (4)	18550	17237	14509	6717	14253
C-202/C-203 (0.8)	3399	1545	688	102	1434

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[0560] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, descriptions and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

[0561] All patents, patent applications, and publications cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent or patent application were specifically and individually indicated to be so incorporated by reference.